AF1q enhancement of γ irradiation-induced apoptosis by up-regulation of BAD expression via NF-κB in human squamous carcinoma A431 cells

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Received September 2009; Accepted December 30, 2009

DOI: 10.3892/or_00000891

Abstract. BAD (BCL-2 antagonist of cell death) is a pro-apoptotic BCL-2 family protein that plays a critical role in the regulation of apoptotic response. This study presents direct evidence that AF1q increased the radiation-induced apoptosis through up-regulation of BAD in human squamous carcinoma A431 cells and the key transcription factor involved is NF-κB. The minimal promoter sequence of *BAD* was identified; the activity was increased in AF1q stable transfectants and decreased upon AF1q siRNA transfection. The NF-κB consensus binding sequence is detected on *BAD* promoter. Inactivation of NF-κB by NF-κB inhibitor Bay 11-7082 or NF-κB p65 siRNA suppressed the expression and promoter activity of *BAD*; the suppression is more obvious in AF1q stable transfectants which also have an elevated NF-κB level. Mutation of putative NF-κB motif decreased the *BAD* promoter activity. The binding of NF-κB to the *BAD* promoter was confirmed by chromatin-immunoprecipitation. These findings indicate that AF1q up-regulation of BAD is through its effect on NF-κB and this may hint of its oncogenic mechanism in cancer.

Introduction

Radiation-induced apoptosis has been shown to proceed via different pathways and one of the critical steps is the permeabilization of outer mitochondrial membrane. The permeabilization is regulated at least by the balance of the expressions of pro- and anti-apoptotic BCL-2 family proteins (1). The alteration of the balance may lead to the leakage of cytochrome *c* and the formation of apoptosome which subsequently activates the caspase cascade culminating in apoptosis (2). BAD (BCL-2 antagonist of cell death) is a BH3-only pro-apoptotic BCL-2 family protein and plays a critical role in radiation-induced apoptosis (3-5). Under physiological conditions, anti-apoptotic BCL-2 family proteins promote cell survival by sequestering the pro-apoptotic BCL-2 proteins such as BAX and BAK (6). To induce apoptosis, BAD binds with the anti-apoptotic BCL-2 proteins (e.g., BCL-2, BCL-xL and BCL-w) to release BAX and BAK (7-9). On the other hand, BAD is also hypothesized to act as a sensitizer that displaces the activator BH3-only proteins (e.g., BIM or tBID) from the anti-apoptotic BCL-2 proteins. The released BIM or tBID activates the oligomerization of BAX and BAK and thus elicits permeabilization of the outer mitochondrial membrane and release of cytochrome *c* (10-12). Recently, it has been demonstrated that BAD can direct p53 to the mitochondria and activate p53-dependent apoptosis (13). The apoptotic activity of BAD is regulated extensively by its phosphorylation at the regulatory serines (Ser^{112}, Ser^{136} and Ser^{155}) through a group of protein kinases such as RSK, Akt and PKA (4,14-17). Phosphorylated BAD forms a complex with 14-3-3 proteins and sequestrates in the cytoplasm, resulting in inhibition of apoptosis (4). However, the regulation of BAD expression is so far not clear.

AF1q is an oncogenic factor involved in hematological malignancy, thyroid tumorigenesis and breast cancer metastasis (18-23). The AF1q gene was initially identified as a mixed lineage leukaemia (MLL) fusion partner from an infant acute myelomonocytic leukemia carrying the t(1;11)(q21;q23) translocation (19). Elevated AF1q expression was reported in pediatric acute myeloid leukemia (AML), myelodysplastic syndrome and thyroid oncocytic tumors (18,20,23). Overexpression of AF1q was also suggested to promote metastasis in human breast cancer (21,22). Our previous studies provide the first evidence that AF1q enhanced the apoptosis induced by several therapeutic agents including doxorubicin, inter-ferons as well as γ irradiation (24). The enhancement of doxorubicin-induced apoptosis by AF1q is subsequently found to be through the up-regulation of BAD.

In the present study, we demonstrated that AF1q increased the radiation-induced apoptosis through up-regulation of BAD. By truncation analysis of the upstream sequence of *BAD* gene, we have identified the minimal promoter sequence of *BAD* and demonstrated that NF-κB is a key transcription factor participating in AF1q up-regulation of BAD and also enhancement of radiation-induced apoptosis.

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Key words: γ irradiation, apoptosis, NF-κB, squamous carcinoma

Materials and methods

Cell culture and reagents. Human squamous cell carcinoma A431 parent (AP) cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen) and incubated in humidified incubator with 10% CO_2 at 37˚C. AP cells stably transfected with vector carrying full-length AF1q cDNA (AQ1 and AQ5) or vector alone (AN) were cultured under the same condition as AP cells, except the medium was supplemented with 300 μg/ml G418 (Calbiochem).

Annexin-V binding assay. Cells were trypsinized, washed and resuspended in Annexin V binding buffer, pH 7.4 (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$). Annexin V-GFP and propidium iodide (PI) were then added to the cell suspension. After incubation at room temperature for 15 min, cells were analysed by flow cytometry FACSCanto (BD Biosciences) and the data obtained was analyzed by WinMDI 2.8.

Quantitative RT-PCR. Total RNA was extracted by lyzing cells with TRI reagent (Molecular Research Center). Total RNA (1.5 μg) was used to synthesize the first strand cDNA by M-MLV reverse transcriptase (Promega). Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR system and the Power SYBR Green PCR Master Mix (Applied Biosystems) under the standard thermal-cycling condition (95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 1 min). The following primers were used: BAD (sense, 5'-AGGGAGGGCTGACCCAGAT-3'; antisense, 5'-GGCGGAAAACCCAAAACTTC-3'), NF-κB p65 (sense, 5'-CCAACAACAACCCCTTCCAA-3'; antisense, 5'-AGAG CCGCACAGCATTCAG-3') and ß-actin (sense, 5'-ACACC CCAGCCATGTACGTT-3'; antisense, 5'-TCACCGGAGTC CATCACGAT-3'). Relative Standard Curve Method ($2^{-\Delta\Delta Ct}$) was used to determine the relative mRNA expression by using ß-actin as the reference.

Western blot analysis. The cells were washed thrice with ice-cold PBS and lysed in Laemmli's lysis buffer. The proteins were separated on SDS-polyacrylamide gels and electrophoretically transferred to Immobilon PVDF membrane (Millipore). The membranes were incubated with primary antibodies against AF1q (Abnova Corp., Taipei City, Taiwan), ß-actin (Sigma), BAD (Santa Cruz Biotechnology), NF-κB p50 and p65 (Cells Signaling) for 2 h followed by appropriate horseradish peroxidase-conjugated secondary antibodies (Zymed) for 2 h at room temperature. Signals were developed by using ECL chemiluminescence detection reagents (Amersham Biosciences) and visualized on X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometric analysis was done using ImageJ (NIH). The band intensity was first normalized with that of ß-actin and then normalized to that of the control sample, of which the expression is designated as 1.0.

siRNA transfection. The AF1q siRNA expression vectors were constructed using p*Silencer* neo siRNA Expression Vector kit (Ambion). Two complementary oligonucleotides (5'-GATCCAGACAGCAGCGTTGGCAAATTCAAGA GATTTGCCAACGCTGCTGTCTTTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAAAGACAGCAGCGTTGGCAA ATCTCTTGAATTTGCCAACGCTGCTGTCTG-3') were synthesized, annealed and ligated into the pSilencer 2.1-U6 Neo vector.

The NF-κB p65 siRNA (sense, 5'-GCCCUAUCCCUUU ACGUCAdTdT-3'; antisense, 5'-UGACGUAAAGGGAUAG GGCdTdT-3') (25) and BAD siRNA (sense, 5'-GAAGGGAC UUCCUCGCCCGdTdT-3'; antisense, 5'-CGGGCGAGGAA GUCCCUUCdTdT-3') together with a negative control siRNA were chemically synthesized by Tech Dragon Ltd. (Hong Kong SAR, China). For transfection, siRNA was allowed to form complexes with Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations.

Promoter constructs and site-directed mutagenesis. A series of human *BAD* gene promoter constructs was obtained by PCR cloning from genomic DNA isolated from human A431 parent cells. Five forward primers (-1722, 5'-ACCGAGCTC GCGCTCAGGAGGAAAGAAGT-3'; -1332, 5'-ACCGAGC TCCTGCCTCCTGTCCTCGTAAT-3'; -1125, 5'-ACCGAG CTCGCCGTTCGCTACGCAAAT-3'; -801, 5'-ACCGAGCT CCCGCCCATAGCCAAGATG-3'; -375, 5'-ACCGAGCTC CCGCAGTAATCACTCCCTTC-3') and one common reverse primer (+2, 5'-CGGGCTAGCTCCGGGCCCTAGTT GCTT-3') were used to generate five progressive deletion constructs. PCR products were subsequently cloned into the luciferase reporter vector pGL3-Basic (Promega). Site-directed mutagenesis was carried out to introduce mutations into the putative NF-κB binding site at position -480 from pGL-1332 using the QuikChange II site-directed kit (Stratagene) using the mutation-containing primers (sense, 5'-GAGGAGCGGG GAGCGTTCCTTTCCGACCGGGCA-3'; antisense, 5'-TGC CCGGTCGGAAAGGAACGCTCCCCGCTCCTC-3').

Transient transfection and luciferase reporter gene activity assay. A431 ($7x10^4$) cells were seeded into 24-well plates one day before transfection. The cells were transfected with 0.8 μg of luciferase-reporter vector containing the BAD upstream genomic sequence using Lipofectamine 2000 (Invitrogen) and pGL3 empty vector was used as a negative control. pRL-CMV (40 ng), which encoding *Renilla* luciferase, was included in all transfections to normalize transfection efficiency. Twenty-four hours after transfection, the cells were washed and lysed with the passive lysis buffer from the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured in each cell lysate using a FLUOstar Galaxy plate reader. Firefly luciferase activity was normalized to *Renilla* luciferase and the data are expressed as fold induction relative to that of the empty pGL3 Basic plasmid.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using the Chromatin Immunoprecipitation Assay kit based on the manufacturer's instruction (Upstate). Briefly, cells were fixed in formaldehyde, lysed and sonicated to shear DNA. After centrifugation, the sonicated cell supernatants were diluted with ChIP Dilution Buffer and aliquots of samples were saved as the input DNA for quantization of the amount of total DNA. For immunoprecipitation, 1 μg of NF-κB p65 antibody or normal rabbit IgG was added to

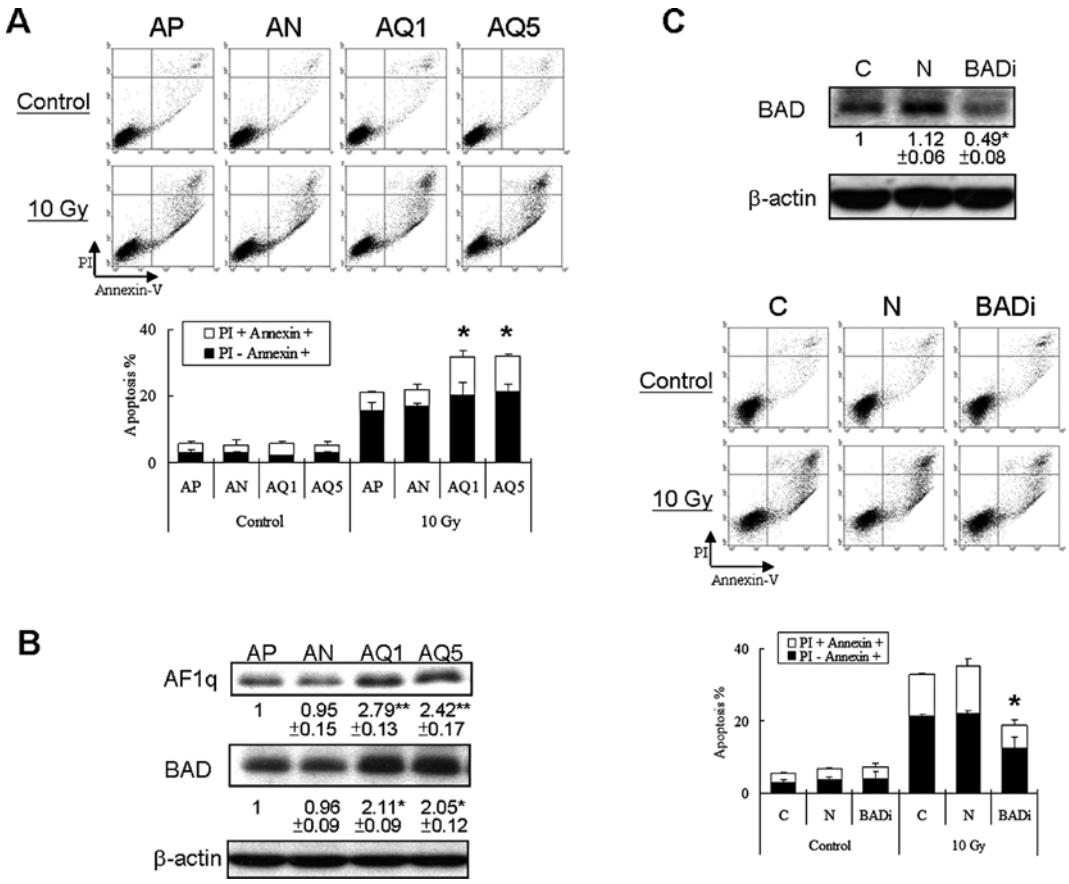

Figure 1. (A) AF1q up-regulation of radiation-induced apoptosis. Subconfluent AF1q stable transfectants were subjected to 10 Gy irradiation. After 48 h, cells were stained with PI and Annexin-V, and then analysed by flow cytometry. The percentage of early apoptotic cells (PI - Annexin-V +, closed bar) and late apoptotic cells (PI + Annexin-V +, open bar) were quantitated and plotted in bar chart. AP, AP cells without transfection; AN, AP cells stably transfected with pcDNA3.0; AQ1 and AQ5, AP cells stably transfected with AF1q-pcDNA3.0. Bars, SD. *P<0.05 (n=3), versus the AN cells. (B) The BAD protein expression in AF1q stable transfectants. The relative protein levels of AF1q and BAD were measured by Western blot analysis. *P<0.05, **P<0.01 (n=3), versus the AN cells. (C) The effect of BAD siRNA transfection on AF1q enhancement of radiation-induced apoptosis. AQ1 cells were transfected with BAD siRNA (BADi) or control siRNA (N) for 24 h. The transfected cells were then lysed for protein extraction or exposed to 10-Gy irradiation. After 48 h, cells were subjected to Annexin-V binding assay. *P<0.05 (n=3), significantly different from cells transfected with control siRNA. C, cells without transfection.

the precleared supernatants and incubated overnight at 4˚C. Immunocomplexes were collected using Protein A Agarose/Salmon Sperm DNA for 1 h at 4˚C. Following the wash, the immunocomplexes were recovered by resuspending in elution buffer. DNA-protein complexes as well as the input DNA were reverse cross-linked at 65˚C for 4 h and treated with proteinase K at 45˚C for 1 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Thereafter, the DNA was subjected to PCR with primers specific for putative NF-κB binding site: forward, 5'-TTCCGCCCATA GCCAAGA-3' and reverse, 5'-GATTACTGCGGGCGA AGG-3'. Amplification was carried out for 35 cycles with denaturation at 94˚C for 30 sec, annealing at 54˚C for 30 sec, and extension at 70˚C for 40 sec.

Results

AF1q enhancement of radiation-induced apoptosis by up-regulation of BAD. To examine the effect of AF1q on radiation-induced apoptosis, AQ1 and AQ5 cells, the two AF1q stable transfectants derived from A431 cells were used. Upon γ irradiation (10 Gy), the apoptotic cells (Annexin-V-positive) in AQ1 and AQ5 cells were 30.23 and 30.03% respectively while there were only 23.07 and 21.89% in A431 parent cells (AP) and cells stably transfected with empty vectors (AN) respectively, indicating AF1q increased the radiation-induced apoptosis in A431 cells (Fig. 1A). BAD was reported to play a critical role in radiation-induced apoptosis (26-28) and was also found to be an important mediator involved in AF1q regulation of doxorubicin-induced apoptosis (24). Therefore, we hypothesized that BAD is also involved in the AF1q enhancement of radiation-induced apoptosis. By Western blot analysis, both the AF1q and BAD protein levels were up-regulated in AQ1 and AQ5 cells when compared with AP and AN cells (Fig. 1B). In addition, after BAD siRNA transfection into AQ1 cells, the apoptotic cells decreased to 21.92% upon irradiation treatment (Fig. 1C). The results therefore suggested that BAD is partici-pated in AF1q enhancement of radiation-induced apoptosis.

BAD is induced by AF1q at the transcription level. By quantitative RT-PCR, BAD mRNA was up-regulated by 2-fold in AF1q stable transfectants (AQ1 and AQ5) when compared with either AP cells or vector control AN cells

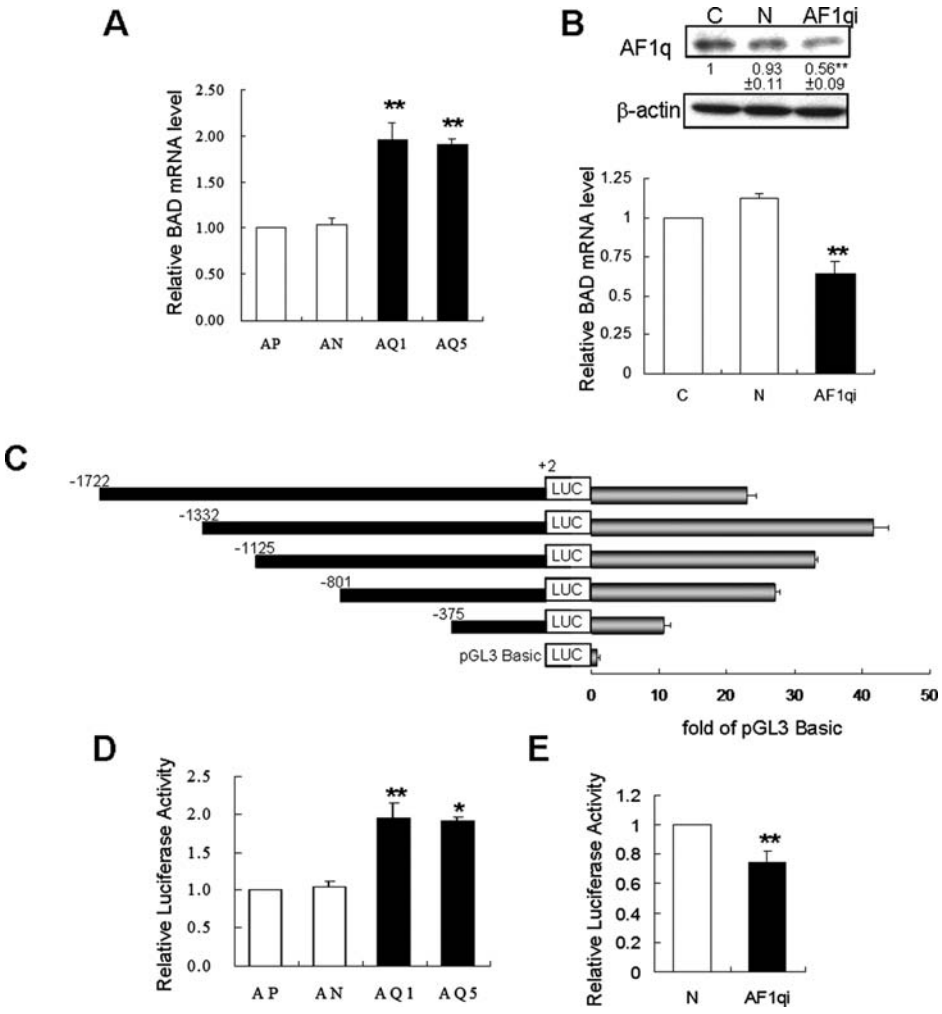

Figure 2. (A) AF1q stable transfection increases BAD mRNA level in cells. BAD mRNA level in AP, AN, AQ1 and AQ5 cells was measured by quantitative RT-PCR. (B) Knock-down of AF1q decreases BAD mRNA level. AP cells were transiently transfected with empty (N) or AF1q siRNA vectors (AF1qi) for 24 h. (C) Analysis of *BAD* promoter region in A431 cells. Schematic view of truncated *BAD* promoter constructs cloned upstream to the firefly luciferase reporter gene (LUC) as shown in the left panel. The numbers represent the distances from the transcription start site (+1). The promoter activity of each construct is presented as fold induction of the empty pGL3 Basic plasmid. (D) *BAD* promoter activity increased in AF1q stable transfectants. (E) AF1q siRNA transfection decreased *BAD* promoter activity. Bars, SD. $^{*}P<0.05$; $^{**}P<0.01$ (n=3), compared with AN cells or cells transfected with the empty vectors (N).

(Fig. 2A). Transient transfection with AF1q siRNA also down-regulated BAD mRNA level in AP cells (Fig. 2B). It is therefore believed that AF1q may up-regulate BAD expression at the transcription level. To investigate the transcription regulation of *BAD* by AF1q, a series of 5'-end truncated *BAD* promoter fragments were constructed. Promoter fragments from positions -1722, -1332, -1125, -801 and -375 to the position +2 were cloned upstream of a firefly luciferase reporter gene. These constructs were transiently transfected into AP cells and the basal transcription activity of *BAD* was measured by dual luciferase assay. As shown in Fig. 2C, the reporter activity of fragment pGL-1332 is the highest among the other constructs, about 42-fold increase above the empty pGL3 vector. It indicates that pGL-1332 may possess the critical elements that are necessary for the maximal promoter activity of *BAD*. Furthermore, overexpression of AF1q by stable transfection increased while transient tranfection of AF1q siRNA decreased the reporter activity of pGL-1332 (Fig. 2D and E). Therefore, pGL-1332 was used for the further study on the transcription regulation of *BAD* by AF1q.

NF-κB mediated AF1q up-regulation of BAD. To identify the transcription factors involved in AF1q up-regulation of *BAD*, the *BAD* promoter fragment pGL-1332 was analyzed by using MatInspector, Transcription Element Search Software (TESS) and also MATCH™ 1.0. Consensus binding sequence for transcription factor NF-κB was identified at -471 to -480 bp by all three software. NF-κB consists of homo- or hetero-dimers and the major form is p65/p50 dimer. By Western blot analysis, NF-κB p65 was up-regulated in AF1q stable transfectants AQ1 and AQ5 while a slight increase was also seen for NF-κB p50 (Fig. 3A). Furthermore, the up-regulation of NF-κB p65 was associated with the increase in the nuclear fraction of the protein (data not shown). The results therefore suggested that NF-κB may be one of the transcription factors involved in AF1q regulation of BAD. To test this hypothesis, the NF-κB inhibitor, Bay 11-7082, and NF-κB p65 siRNA were used to inactivate NF-κB in AP and AQ1 cells. By Western blot analysis, Bay 11-7082 down-regulated the BAD protein level in both cells in a dose- and time-dependent manner (Fig. 3B). Transfection with NF-κB

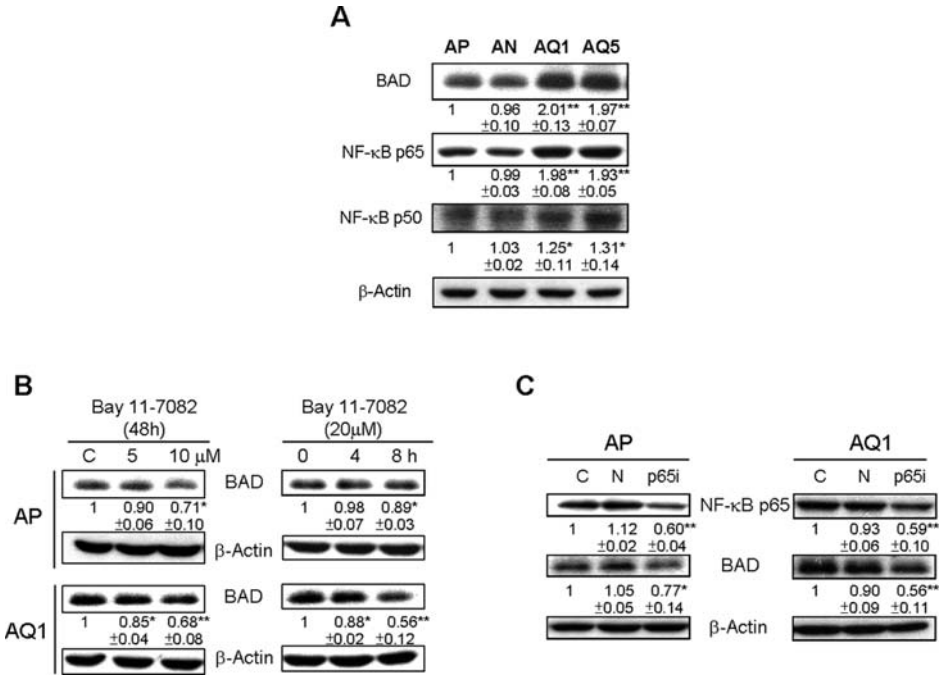

Figure 3. (A) Protein expressions of NF-κB subunits in AP, AN, AQ1 and AQ5 cells. Effect of NF-κB inhibitor Bay 11-7082 (B) and NF-κB p65 siRNA (C) on BAD protein level in cells. (B) AP and AQ1 cells were treated with the indicated concentration of Bay 11-7082 for 48 h or treated for the indicated time with 20 μM Bay 11-7082. *P<0.05; **P<0.01 (n=3), compared with cells with no inhibitor (C) or at time zero (0 h). (C) AP and AQ1 cells were transfected with NF-κB p65 siRNA (p65i) or negative control siRNA (N). *P<0.05; **P<0.01 (n=3), compared with cells transfected with the empty vectors (N).

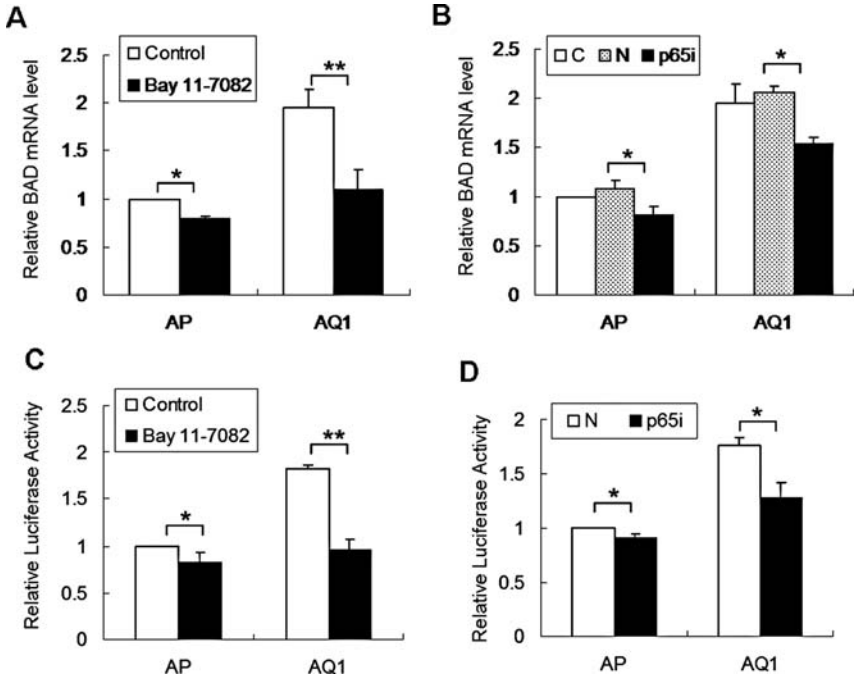

Figure 4. NF-κB inhibitor Bay 11-7082 (A) and NF-κB p65 siRNA (B) decreased the mRNA level of BAD. (A) AP and AQ1 cells were incubated in 20 μM Bay-11-7082 for 8 h and total RNA was extracted for quantitative RT-PCR. (B) Cells were transfected with NF-κB p65 siRNA (p65i) or negative control siRNA (N) for 24 h. NF-κB inhibitor Bay 11-7082 (C) and NF-κB p65 siRNA (D) decreased the BAD promoter activity. (C) Cells were pre-incubated with 20 μM Bay-11-7082 for 2 h. The promoter activity of pGL-1332 was measured. (D) pGL-1332 was co-transfected with NF-κB p65 siRNA (p65i) or negative control siRNA (N) into cells according to the manufacturer's instructions. Bars, SD. *P<0.05; **P<0.01 (n=3), compared with cells without drug treatment (control) or transfected with negative control siRNA (N).

p65 siRNA also decreased the BAD protein expression level in AP and AQ1 cells (Fig. 2C). NF-κB not only affected the protein expression of BAD, but also the expression of BAD mRNA. By quantitative RT-PCR, addition of 20 μM Bay 11-7082 (Fig. 4A) or transfection with NF-κB p65 siRNA (Fig. 4B) suppressed the BAD mRNA level in both AP and

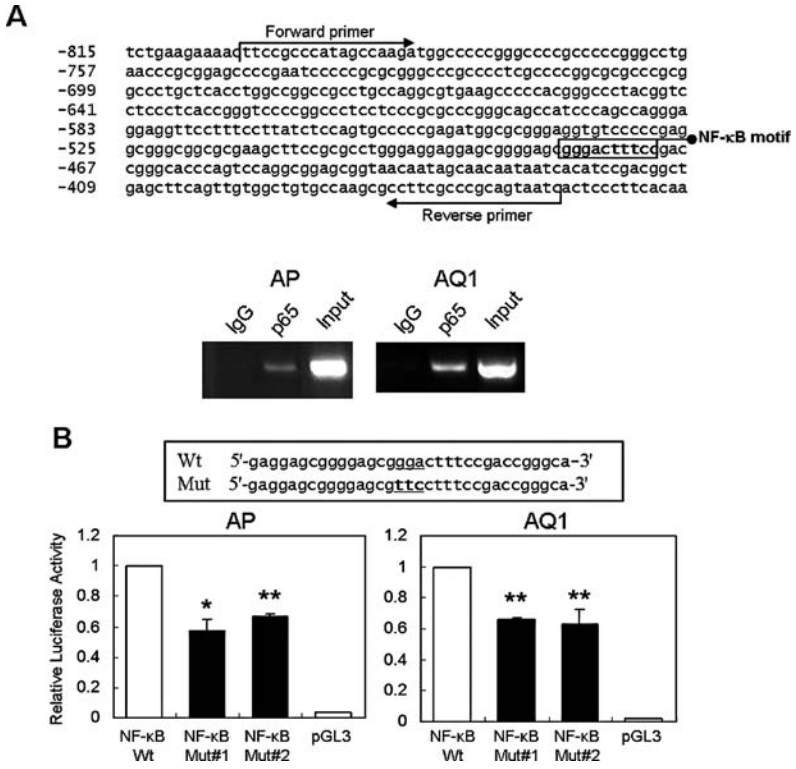

Figure 5. (A) *In vivo* interaction of NF-κB with the *BAD* promoter by ChIP assay. Upper, upstream sequence of *BAD* promoter and location of primers used in the ChIP assay. The putative NF-κB binding motif is highlighted. Bottom, ChIP assay was performed using NF-κB p65 antibody. Normal rabbit IgG was included as the negative control (IgG). The recovered chromatin was subjected to PCR analysis using primers covering the putative NF-κB motif of the *BAD* promoter. A representative experiment is shown, and similar results were obtained from three independent experiments. (B) Mutational analysis of putative NF-κB binding site in the *BAD* promoter. Point mutations were introduced into the *BAD* promoter construct pGL-1332 as indicated and two mutated clones (NF-κB Mut no.1 and no.2) were chosen for the luciferase acitivity assays. Bars, SD. *P<0.05; **P<0.01 (n=3), compared with the wild-type constructs.

AQ1 cells; the suppression effect is greater in AQ1 cells. After the incubation with Bay 11-7082 (Fig. 4C) or co-transfection with NF-κB p65 siRNA (Fig. 4D), the luciferase activity of *BAD* promoter fragment pGL-1332 was also decreased significantly in AQ1 cells and slightly in AP cells. These data indicate that NF-κB may mediate AF1q transcription regulation of BAD.

NF-κB binds to the BAD promoter. To determine if NF-κB binds to the putative NF-κB binding site within the *BAD* promoter *in vivo*, chromatin immunoprecipitation (ChIP) assay was performed using anti-NF-κB p65 antibody. NF-κB p65 bound chromatin was immunoprecipitated from nuclear extracts of AP and AQ1 cells and subjected to PCR analysis using primers covering the putative NF-κB motif, -471 to -480 bp, in the BAD promoter. As shown in Fig. 5A, a 439-bp PCR product was amplified from NF-κB p65-immunoprecipitated DNA samples in both AP and AQ1 cells, but not in the samples with normal rabbit IgG. It indicates that NF-κB binds to the site within this sequence. Moreover, by mutating the putative NF-κB motif (-471 to -480 bp), the luciferase activity of *BAD* promoter pGL-1332 was decreased significantly (Fig. 5B). It further confirmed that NF-κB is involved in the AF1q up-regulation of *BAD* expression. The effect of NF-κB on BAD is also in line with the enhancement role of AF1q in radiation-induced apoptosis. After transient transfection of NF-κB p65 siRNA (p65i), the radiation-induced apoptotic cells in AQ1 cells were decreased significantly from 28.21 to 21.92% (Fig. 6). This result suggested that NF-κB p65 may be the

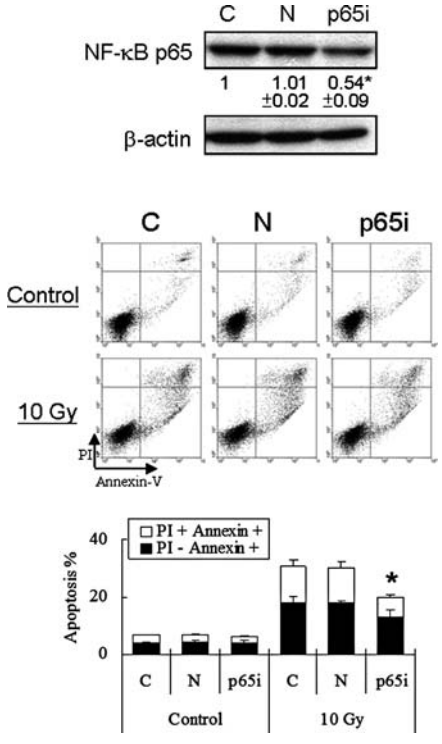

Figure 6. The AF1q enhancement of radiation-induced apoptosis was suppressed by NF-κB p65 siRNA. AQ1 cells was transfected with NF-κB p65 siRNA (p65i) or control siRNA (N) for 24 h. Subsequently, cells were treated with 10-Gy irradiation and subjected to Annexin-V binding assay after 48 h. *P<0.05 (n=3), compared with cells transfected with control siRNA. C, cells without transfection.

downstream mediator of AF1q in regulation of BAD expression and consequently the radiation-induced apoptosis.

Discussion

AF1q may sensitize the radiation-induced apoptosis in A431 cells through up-regulation of BAD. Upon γ irradiation, AF1q stable transfectants AQ1 and AQ5 showed an increase in apoptosis induction when compared with control cells AP and AN (Fig. 1A). The results are in consistent with the previous report that transient transfection of AP cells with antisense AF1q oligonucleotides significantly decreased the gamma irradiation-induced DNA fragmentation (24). The pro-apoptotic protein BAD was up-regulated in AF1q stable transfectants and knock-down of BAD reduced the AF1q enhancement of radiation-induced apoptosis (Fig. 1B and C). We therefore speculated that BAD is the key protein involved in AF1q enhancement of radiation-induced apoptosis.

AF1q regulates BAD expression at the transcription level. The apoptotic activity of BAD is determined largely on its phosphorylation status, mainly at Ser^{112}, Ser^{136} and Ser^{155} (4). Phosphorylation of BAD was, however, found not to be affected by AF1q (24). AF1q up-regulates BAD at protein as well as at mRNA level. The AF1q induction of BAD, however, was inhibited by actinomycin D (data not shown). Therefore, AF1q will likely regulate the transcription of BAD.

NF-κB mediates the AF1q up-regulation of BAD and also the enhancement of radiation-induced apoptosis. Binding of p53 to the 6.6 kb upstream of the transcription start codon of *BAD* gene was previously reported (13). However, AF1q up-regulation of BAD is unlikely p53-dependent as the p53 protein in A431 cells is R273H mutant. The expression of BAD was previously reported to be induced by either etoposide or TRAIL which were also shown to increase the transcriptional activity of NF-κB. The detailed mechanism was however not suggested (29-32). The regulatory effect of NF-κB on BCL-2 family members has been suggested by many investigators. NF-κB can transcriptional activate the promoters of BCL-2, BCL-2 homologues BFL-1, BAX, BCL-XL and its proapoptotic alternatively spliced form, BCL-xS (33-37). By truncation analysis, the minimal promoter sequence of *BAD* (-1332 to +2) was identified in the present study. The consensus binding sequence for transcription factor NF-κB was subsequently located by computational sequence analysis. Mutation of the putative NF-κB motif (-471 to -480 bp) decreased the promoter activity of *BAD* (Fig. 5B). Binding of NF-κB to the *BAD* promoter was indicated by chromatin immunoprecipitation (ChIP) assay (Fig. 5A). Inactivation of NF-κB by either NF-κB inhibitor or NF-κB p65 siRNA decreased not only both the mRNA and protein levels, but also the promoter activity of *BAD* in AF1q stable transfectants, the cells with elevated NF-κB expression. A similar but a lesser reduction was also observed in AP cells, the cells with less NF-κB expression (Figs. 3 and 4). All these results indicated that NF-κB is involved in the AF1q up-regulation of *BAD*. As a result of mediating the AF1q up-regulation of BAD, NF-κB also mediates the AF1q effect on radiation-induced apoptosis as knock-down of NF-κB p65 suppressed AF1q effect on BAD expression as well as radiation-induced apoptosis (Fig. 6).

In conclusion, AF1q was found to increase the protein expression of NF-κB p65 which in turn transactivates *BAD* and enhances the radiation-induced apoptosis. The finding for AF1q regulation of NF-κB p65 may provide insight into the oncogenic mechanism of AF1q as aberrant expression of NF-κB is shown to be associated with the development of various types of cancer (38-40). Furthermore, the sensitization of radiation-induced apoptosis by AF1q indicates its potential to serve as a target in cancer therapy.

Acknowledgements

The study is supported by a grant from Hong Kong Research Grants Council Earmarked Grant CUHK4428/05M.

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