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Fas-induced apoptosis of renal cell carcinoma is mediated by apoptosis signal-regulating kinase 1 via mitochondrial damage-dependent caspase-8 activation

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Abstract. Renal cell carcinoma (RCC) is a prototype of a chemo refractory tumour. It remains the most lethal of the common urologic cancers and is highly resistant to conventional therapy. Here, we confirmed the efficiency of anti-Fas monoclonal antibody (CH11) as alternative therapeutic approach for the treatment of RCC and investigated the molecular mechanism(s), whereby CH11 induces apoptosis of RCC cells. The present study shows an essential role for apoptosis signal-regulating kinase 1 (ASK1), together with both c-jun-N-terminal kinase (JNK) and p38 pathways, and caspase-8 in this process. Furthermore, CH11-dependent induction of the ASK1–JNK/p38 pathways was found to activate the transcription factors AP-1 and ATF-2, and FADD-caspase-8-Bid signalling, resulting in the translocation of both Bax and Bak proteins, and subsequently mitochondrial dysregulation that is characterized by the loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome c release and cleavage of caspase-9, caspase-3 and PARP. Thus, the described molecular mechanisms of CH11-induced apoptosis suggest the reliability of Fas activation as an alternative therapeutic approach for the treatment of patients with advanced renal cell carcinoma.

Keywords: Apoptosis, RCC, CH11, ASK1, MAP kinase

1. Introduction

Renal cell carcinoma (RCC) is a prototype of a chemo refractory tumour, which originates in the renal cortex, and accounts for 80–85% of all malignant tumours of kidney [1]. It remains the most lethal of the common urologic cancers, with approximately 40% of patients eventually dying of disease progression [2]. It is highly resistant to chemotherapy [3–5], and few patients respond to interleukin-2 or interferon (IFN)-based immunotherapy that can be associated with considerable toxicity [6].

Pathologically, failure of an apoptosis program often leads to an imbalance in cell numbers, which in turn can lead to tumorigenesis. Thus, control of apoptosis is considered an important strategy for clinical cancer therapy [7]. While most anti-cancer therapies induce apoptosis, the generation of apoptosis-resistant tumour cells is a major barrier to long-term cancer treatment. Tumour cells that become resistant to one therapy are usually cross-resistant to subsequent thera-

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pies, including those with different cellular/molecular targets, suggesting that resistant tumour cells acquire modifications of the general apoptotic pathway. Therefore, *in vitro* analysis of therapeutic approaches up on their clinical relevance is important for patients with advanced tumours, including RCC.

Apoptosis plays a pivotal role during embryonic development, tissue remodelling, immune regulation, and tumour regression [8]. Although there are multiple agents that can induce apoptosis, the best characterized are the death-inducing members including CD95L [9,10], which is extremely efficient in killing a variety of tumour cells. Importantly, the cross-linking of CD95 with anti-CD95 monoclonal antibody (mAb) has been shown to cause apoptosis in CD95-bearing cells [11,12].

Mitogen-activated protein (MAP) kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction cascades that play a regulatory role in cell growth, differentiation and apoptosis [13–15]. MAP kinases are activated in response to their phosphorylation within a TXY motif, by dual specificity MAP kinase kinases (MAPKKs). A number of MAPKKs have been identified as upstream activators of the JNK and p38 pathways, including ASK1 [16–18]. Activation of the JNK and p38 signalling pathways leads to phosphorylation of a number of targets including the transcription factors ATF-2 and AP-1 [19–21] resulting in an increase in their transcriptional activity.

Based on the apoptotic potential of anti-Fas mAb (CH11) and the broad sensitivity of many tumour types to this agent, we evaluated, *in vitro*, CH11-induced cell death in an established human renal cell carcinoma model and investigated the molecular mechanism(s), which are responsible for the regulation of CH11-induced apoptosis of RCC cells.

In this study, we provide evidence for the validity of CH11 in the treatment of RCC and set out for the first time the importance of ASK1–JNK/p38 pathways in the modulation of Fas-induced pathways to apoptosis in RCC cells by a mechanism including mitochondrial damage-dependent caspase-8 activation.

2. Material and methods

2.1. Assessment of cell survival

RCC cell lines ACHN and Caki-1 were obtained from the American Tissue Culture Collection (ATCC), Rockville, MD, were seeded in 96-microwell plates (1×10^4 cells/well). 24 h later, the cells were exposed to the agonistic anti-Fas antibody, CH11 (250 ng/ml) (Immunotech, Marseille, France) for the indicated time periods. The percentage of viable cells was then determined using the colorimetric MTT assays (Roche, Mannheim, Germany) as described [22,23].

2.2. qRT-PCR

Total RNA was isolated from Caki-1 and ACHN cells before and after the treatment with CH11 using RNeasy kit (Qiagen). Reverse transcription was performed using high-capacity cDNA Archive kit (Applied Biosystems, Weiterstadt, Germany). Primer sequences for Bax were 5′-ATG GAC GGG TCC GGG GAG-3′ forward and 5′-TCA GCC CAT CTT CTA GAT-3′ reverse, and Primer sequences for β-actin were 5′-TGA GGC ACT CTT CCA GCC TT-3′ forward and 5′-CAC TTC ATG ATG GAG TTG AAG GTA GT-3′ reverse. Data were analysed according to the comparative Ct method and were normalized by β-actin expression in each sample as described [24,25].

2.3. Flow cytometry

Immunostaining of intact and permeabilized cells was carried out as described [19,22]. Analysis was carried out by a FACSCalibur (Becton Dickinson Biosciences, Heidelberg, Germany) using anti-Bax (Clone YTH-6A7) (Trevigen, Gaithersburg, MD, USA) or anti-Bak antibody (Clone Ab-1) (Oncogene Research Products, Boston, MA, USA). The percentage of antigen positive cells was calculated as the difference in positive area between the positive and negative control histograms. The positive area was that to the right of the intersection of the two curves.

2.4. Detection of apoptosis by annexin V/PI-staining

Flow cytometric analysis of apoptosis was performed using FACSCalibur (Becton Dickinson Biosciences, Heidelberg, Germany). The staining of treated and control cells by annexin V/PI (Invitrogen, Karlsruhe, Germany) was done as described [19,22].

2.5. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) using JC-1

Flow cytometric analysis of mitochondrial membrane potential was assessed by FACSCalibur (Becton

Dickinson Biosciences, Heidelberg, Germany). The staining of treated and control cells by JC-1 (Invitrogen, Karlsruhe, Germany) was performed as described [19,22].

3. Immunoblot

Immunoblot analysis was performed according to standard procedures using the following antibodies and dilutions: ASK1 (Sc-7931), 1:2000; JNK (Sc-474), 1:2000; p38 (Sc-535), 1:2000; ERK1/2 (Sc-154-G), 1:2000; Actin (Sc-1615), 1:5000; Bid (Sc-6539), 1:500 (each Santa Cruz Biotechnology, CA, USA); Bax (#2772) 1:1000; Bak (#3814), 1:1000; caspase-3 (#7190), 1:1000; caspase-9 (#9501), 1:1000; caspase-8 (#9496), 1:1000; PARP (#9542), 1:500; FADD (#2782), 1:1000; phosphor-FADD (#2785), 1:500 (each Cell Signaling Technology Inc., Danvers, MA, USA) and anti-cytochrome c (ab1357-100), 1:1000 (Abcam plc, Cambridge, UK).

4. *In vitro* kinase assay

In vitro kinase assay was performed as described [19–22]. Briefly, Caki-1 and ACHN cells were treated with CH11 (250 ng/ml). The cells were harvested at the indicated time points and the total cell lysates were prepared using 500 µl of buffer L (20 mM HEPES [pH 7.9], 10 mM EGTA, 40 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM DTT, 1% NP-40, 5 µg apoprotinin, 1 mM leupeptin, 1 µg/ml, pepstatin and 1 mM benzamidine). Insoluble material was removed by centrifugation, and the cell lysate was incubated with specific antibodies to ASK1 (Sc-7931), JNK (Sc-474), p38 (Sc-535) (all from Santa Cruz) for 1 h at 4°C. The immune complexes were bound to A-sepharose (5 mg/ml in lysis buffer) by rotating overnight at 4°C. After centrifugation, the sepharose beads were washed three times with kinase reaction buffer (80 mM HEPES [pH 7.9], 80 mM $MgCl_2$, 0.1 mM ATP, 2 mM Na_3OV_4 and 20 mM NaF). Kinase activity was determined by incubation with 2 µg of GST-c-Jun (Santa Cruz) protein as substrate for JNK or MBP (Biomol GmbH, Hamburg) protein as substrate for ASK1 and p38, and 10 µCi of [γ-^{32}P]dATP (Hartmann Analytika; Munchen) in 15 µl of kinase reaction buffer and then incubated for 30 minutes at 37°C. Reactions were terminated by addition of 15 µl of sample buffer and analysed by SDS-polyacrylamide gel electrophoresis. The gel was dried and autoradiographed.

5. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed as described [19–22]. Briefly, Double stranded synthetic oligonucleotides carrying binding sites for ATF-2 (5′-GTT GAC GTC ACA-3′), AP-1 (5′-TGAC TCA-3′) (all from Santa Cruz) were end-labelled with [γ-^{32}P] dATP (Hartmann Analytika) in the presence of T4 polynucleotide kinase (GeneCraft, Germany). For binding, 4 µg nuclear extract was bound to a 0.2 ng probe in a total volume of 30 µl for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.5; 50 mM NaCl, 1 mM EDTA; 1 mM $MgCl_2$; 0.5 mM DTT and 4% glycerol). The specificity of binding was analysed by competition with an unlabeled oligonucleotide as well as with super shift assay. The competition assay was performed in the same manner, except that unlabeled probes containing either ATF-2, AP-1 sequences, were incubated with nuclear extracts for 20 min at room temperature before adding the labelled probes. The supershift assays were performed by incubation the nuclear extracts with antibodies to either anti-ATF-2 antibody (Sc-242), or anti-c-jun antibody, a (Sc-1694) (all from Santa Cruz), to prove the specificities of ATF-2 and AP-1 DNA-binding activities, respectively. The antibodies were pre-incubated for 30 min at 37°C and then subjected to EMSA as described for ATF-2 and AP-1. Electrophoresis was performed for 3 h at 100 V in 0.5 X Tris-borate-EDTA running buffer at room temperature. The dried gel was visualized by exposure to high performance autoradiography film.

6. Caspase activity assay

Caspase activity was measured using a fluorometric substrate assay as described [22]. Briefly, lysates from treated and control cells were supplemented with 50 mM of the fluorogenic substrates DEVD-AMC for caspase-3 Ac-IETD-AFC for caspase 8 and Ac-LEHD-AFC for caspase-9 (BD Bioscience San Diego, USA). The release of aminomethylcoumarin was measured fluorometrically over 5 h at 37°C using a Lambda Fluro 320 Plus fluorometer (Biotek, Bad Friedrichsall, Germany; excitation: 360 nm, emission: 475 nm). The catalytic activities are expressed as fluorogenic units (FU/min). The caspase-8 inhibitor Ac-IETD-CHO was purchased from MP Biomedicals and used at a concentration of 50 mM.

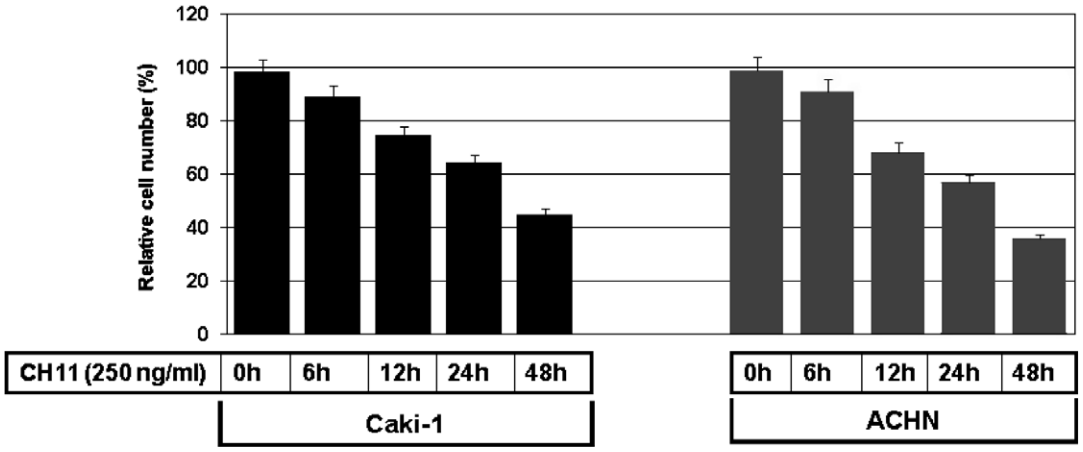
7. RNA interference

SMARTpool® ASK1 siRNA and control siRNA (Scramble siRNA) all from Dharmacon Research, Lafayette, Co., USA. Cells were transfected with lipofectamine 2000 according to the manufacturer's protocol before the exposure of cells to CH11 as described [22].

8. Results

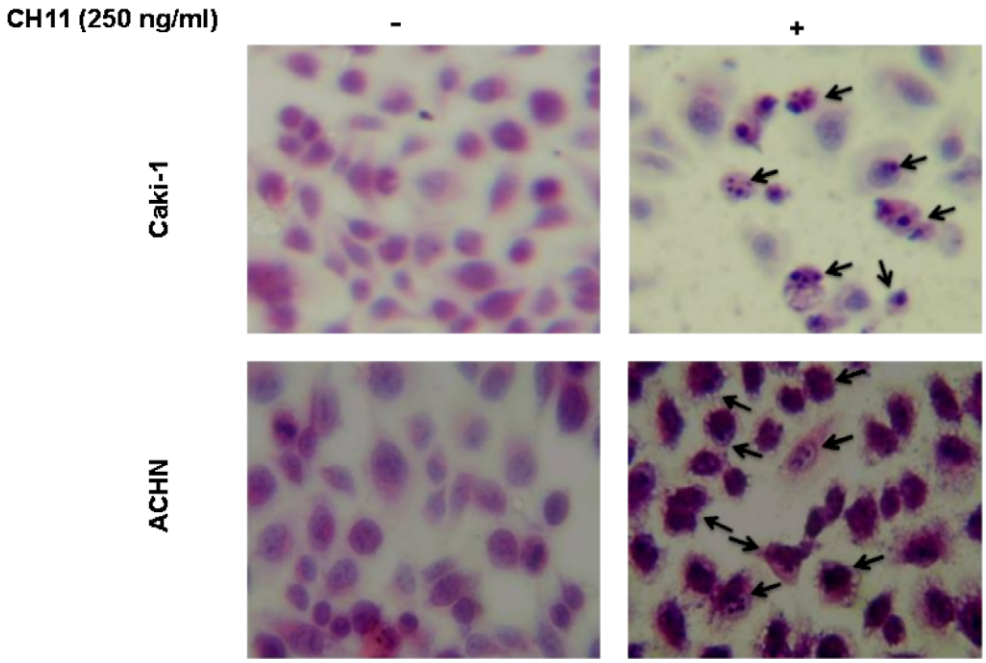
8.1. Sensitivity of human RCC to Fas-mediated apoptosis

Based on the apoptotic efficiency of anti-Fas antibody (CH11) and the broad sensitivity of many tumour types to this agent [26–28], we evaluated its killing efficiency in human renal cell carcinoma (RCC) derived cell lines. The cell lines Caki-1 and ACHN were exposed to CH11 for regular time intervals up to 48 h, after which the cell viability was determined using MTT assay. The reduction of cell viability was noted within 12 h in both RCC cell lines after the exposure to CH11 and increased thereafter up to 48 h (Fig. 1(A)). Furthermore, morphological changes, including rounding up, nuclear condensation and fragmentation, were clearly observed in CH11-treated RCC cells by H&E staining (Fig. 1(B)). To confirm whether CH11-induced death of RCC cells is mediated through an apoptotic mechanism, the cells were treated with CH11 for 48 h, after which the cells were subjected to flow cytometry analysis using Annexin V/PI (for the evaluation of apoptosis) or JC-1 staining (for the measurement of $\Delta\Psi_m$). The analysis of Annexin V/PI stained cells (Fig. 1(C)) demonstrated the induction of apoptosis in Caki-1 (35%) and ACHN (19%) cells by the treatment with CH11 for 48 h. Furthermore, data obtained from flow cytometry analysis of JC-1 stained cells (Fig. 1(D)) showed a significant loss of $\Delta\Psi_m$ after the exposure to CH11. To confirm whether CH11-induced both cell death and changes of $\Delta\Psi_m$ are associated with cytochrome c release and cleavage of caspase-9, caspase-3 and PARP, lysates of CH11-treated cells were subjected to Western blot analysis. Figure 1(E) demonstrates the release of cytochrome c, and the cleavage of caspase-9, caspase-3, and PARP in CH11-treated cells, suggesting a potential role for mitochondria in the modulation of CH11-induced apoptosis of RCC cells. Next, we asked whether CH11-induced



(A)

Fig. 1. Fas-induced apoptosis in RCC cells. (A) MTT assay demonstrating the relative cell number (%) of Caki-1 and ACHN cells after the treatment with CH11 (250 ng/ml) for regular time intervals up to 48 h. Data presented are the mean ± SD of three independent experiments. (B) Detection of apoptosis in CH11-treated cells using H&E staining. Arrows show nuclear condensation, apoptotic blebs and fragmentation of apoptotic cells (magnification ×40). (C) Detection of apoptosis in CH11-treated by flow cytometry using Annexin V/PI. (D) Measurement of CH11-induced changes of $\Delta\Psi_m$ by flow cytometry using JC-1 staining. (E) Detection of cytochrome c release, cleavage of caspase-9 and 3, and PARP by Western blot analysis in CH1-treated cells. (F) Western blot analysis of both total protein expression and phosphorylation of FADD, and the cleavage of both Bid and caspase-8 in RCC cells treated with CH11. β-actin was used as internal control for loading and transfer. Data are representative of three independent experiments.



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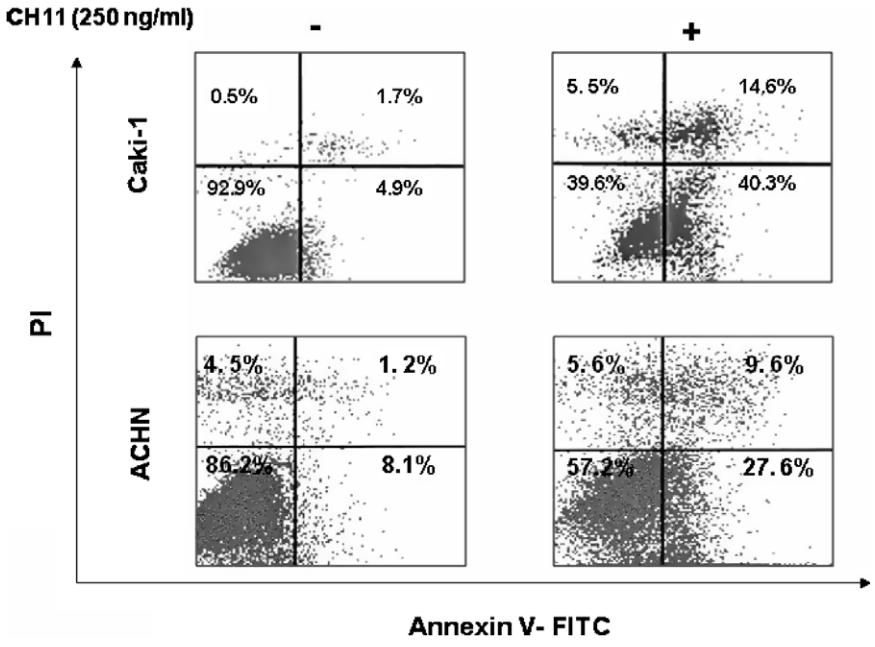
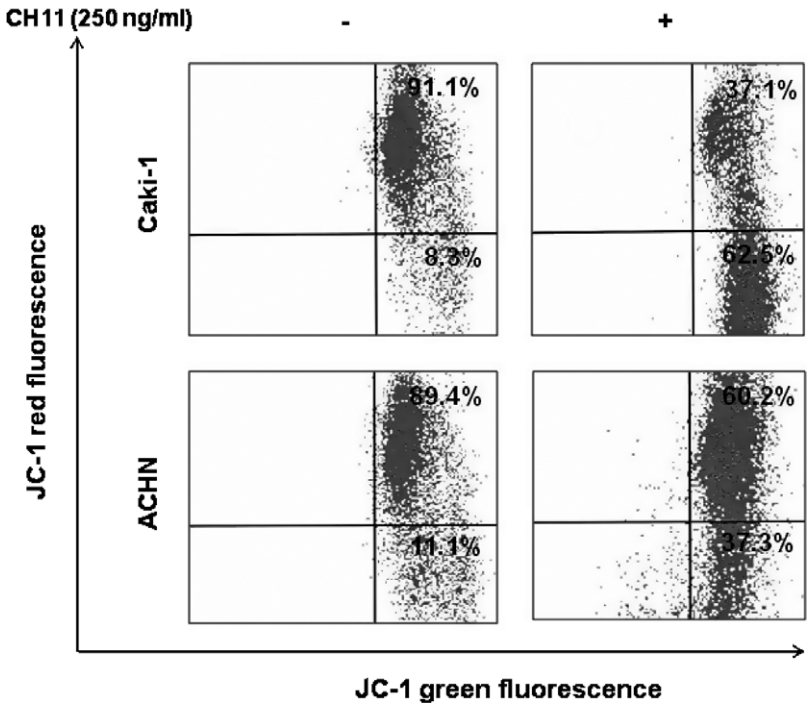


(C)

Fig. 1. (Continued).

apoptosis in RCC cells is associated with the phosphorylation of FADD and/or the activation of caspase-8 and subsequently the cleavage of Bid. Interestingly, data obtained from Western blot analysis of lysates prepared from CH11-treated cells (Fig. 1(F)) demonstrated the phosphorylation of FADD and the cleavage of both caspase-8 and Bid in response to the treatment with CH11, suggesting an important role for FADD and caspase-8 in the regulation of CH11-induced apoptosis of RCC cells.



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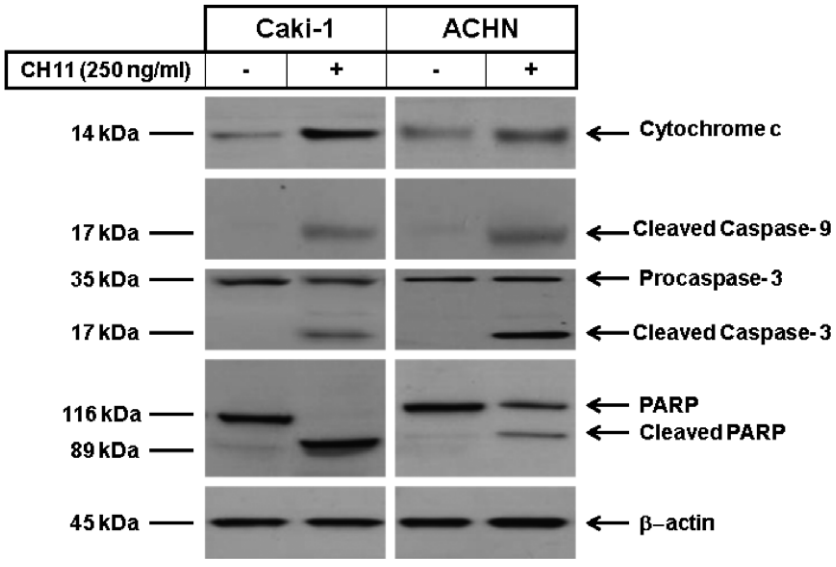
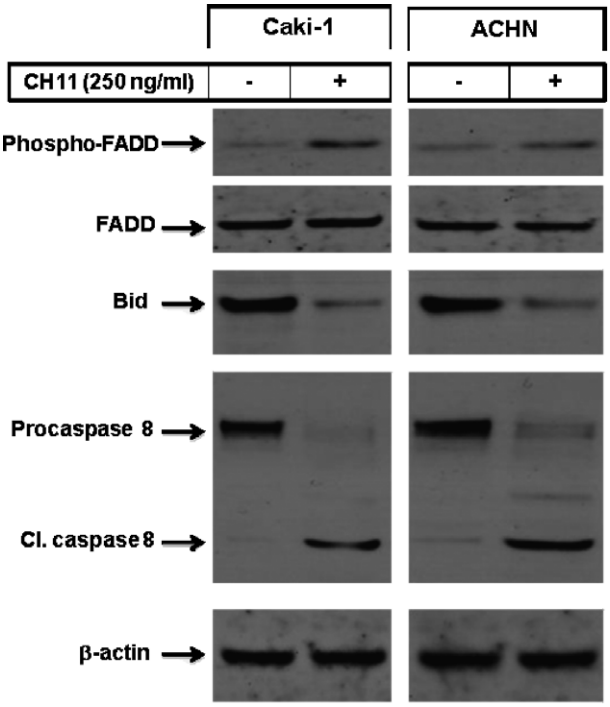


(E)

Fig. 1. (Continued).

8.2. *Activation of ASK1, JNK and p38 pathways and their physiological substrates AP-1 and ATF-2 during CH11-induced apoptosis*

To investigate whether the treatment of RCC cells with CH11 influences the expression and/or the activation of ASK1 and its downstream signalling pathways including JNK, p38 and ERK, both Caki-1 and ACHN cells were treated with CH11 for 48 h, and the total cell lysates were subjected to Western blot analysis or *in vitro* kinase assay. Data obtained from Western Blot analysis (Fig. 2(A)) demonstrated no al-



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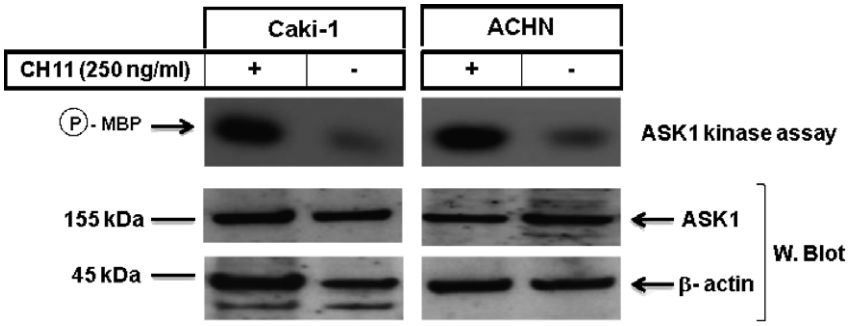
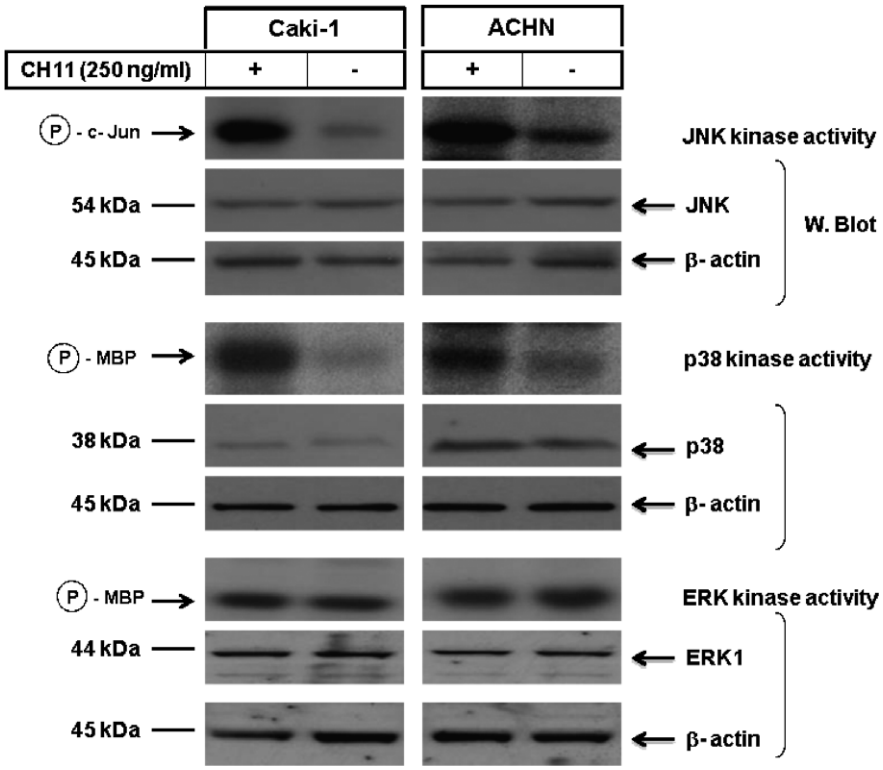


(A)

Fig. 2. Activation of ASK1, JNK, p38, AP-1 and ATF-2 in CH11-treated cells. (A) Western blot analysis and *in vitro* kinase assay of ASK1 before and after the treatment with CH11. (B) Western blot analysis and *in vitro* kinase assay of JNK, p38 and ERK before and after the treatment with CH11. Results are representative of four separate experiments. β-actin was used as internal control for loading and transfer. (C, D) Activation of the transcription factors AP-1 and ATF-2 by CH11. The DNA-binding activities of both AP-1 (C) and ATF-2 (D) were determined by EMSA. The specificities of the binding activity of AP-1 and ATF-2 were analysed by competition with unlabelled oligonucleotides. Results are representative of four independent experiments.

teration at the expression level of ASK1 in CH11-treated cells, whereas, *in vitro* kinase assays (Fig. 2(A)) demonstrated the activation of ASK1 in RCC cells by the treatment with CH11. Next, we examined the effect of CH11 on both expression and activation of MAP kinase signalling pathways JNK, p38 and ERK in CH11-treated and control cells. Although no change was noted at the protein level of the three MAP kinases JNK, p38 and ERK, *in vitro* kinase assay (Fig. 2(B)) revealed a significant increase of the basal activities of JNK and p38, but not the activity of ERK pathways. These results suggest an important role for ASK1–JNK/p38 pathways in the modulation CH11-induced apoptosis in RCC cells.



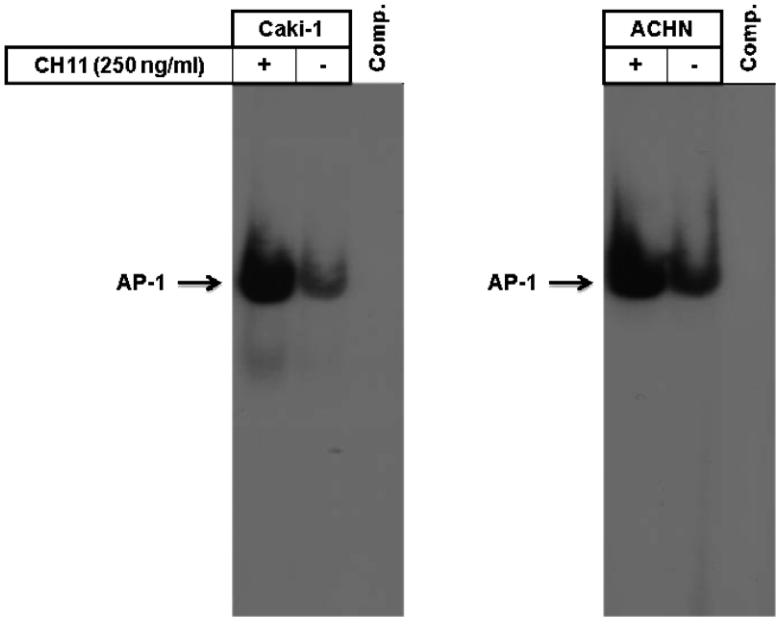
(B)

Fig. 2. (Continued).

To investigate whether the activation of JNK and p38 pathways is implicated in the regulation of their physiological substrates AP-1 and ATF-2, respectively, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from CH11-treated and control cells. EMSA analysis (Fig. 2(C, D)) demonstrated the enhancement of the DNA-binding activities of both AP-1 (Fig. 2(C)) and ATF-2 (Fig. 2(D)) in response to the treatment with CH11. The specificities of AP-1 complex (Fig. 2(C)) and ATF-2 complex (Fig. 2(D)) were demonstrated by supershift assays. Thus, the treatment of RCC cells with CH11 leads to the activation of the transcription factors AP-1 and ATF-2 by their upstream signalling pathways JNK and p38, respectively.

8.3. *Essential role of ASK1, and both JNK and p38 pathways for CH11-induced apoptosis in RCC cells*

We next analysed the role of ASK1 in Fas-mediated pathways to apoptosis in RCC cells. Both Caki-1 and ACHN cells were challenged with 250 ng/ml CH11 for 48 h after the transfection with ASK1-specific siRNA. Cell lysates were subjected to Western blot analysis, *in vitro* kinase assay, as well as to flow cytometry analysis using Annexin V/PI. We first confirmed the efficiency of ASK1 down regulation after transfection with ASK1-specific siRNA. As shown in Fig. 3(A), the transfection with siRNA significantly reduced the level of ASK1 protein. In accordance with the knockdown of ASK1, *in vitro* kinase assay (Fig. 3(A)) demonstrated the inhibition of CH11-induced activation of both JNK and p38 pathways. Also, the effect of the knockdown of ASK1 protein on the extension of CH11-induced apoptosis was examined by flow cytometry analysis using annexin V/PI. Figure 3(B) demonstrates the inhibition of CH11-induced apoptosis in response to the knockdown of ASK1 by its specific siRNA. To evaluate whether the inhibition of JNK and p38 pathways by their specific inhibitors would block CH11-induced apoptosis, both Caki-1 and ACHN cells were pretreated with the inhibitor of JNK (SP600125) and/or the inhibitor of p38 (SB-203580) and the extension of apoptosis was determined by flow cytometry using Annexin V/PI. The flow cytometry data (Fig. 3(C)) re-



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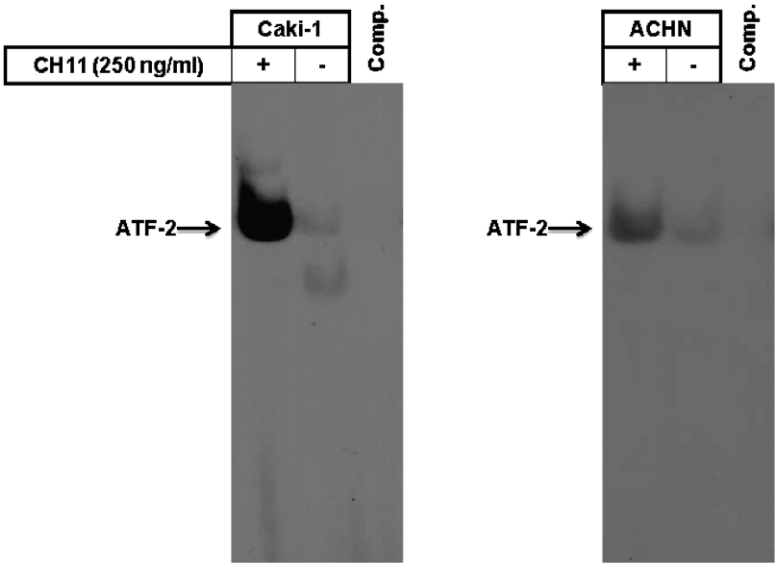
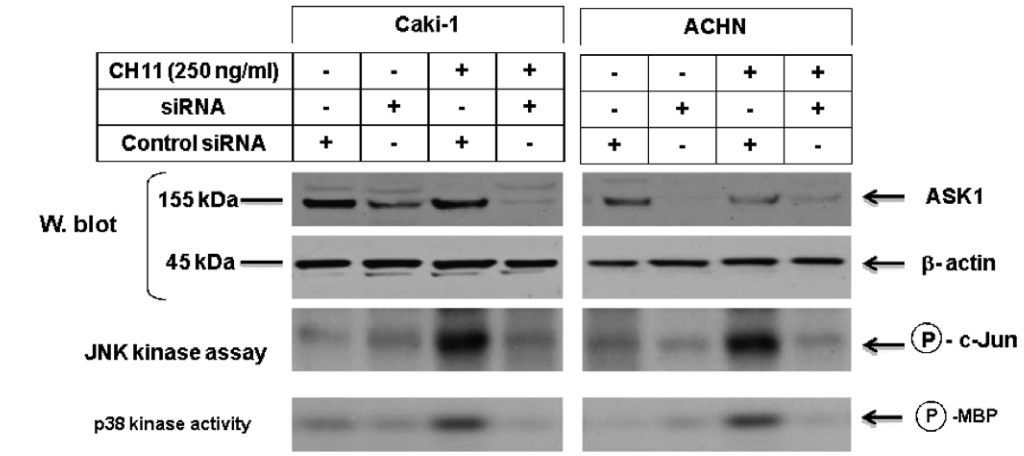


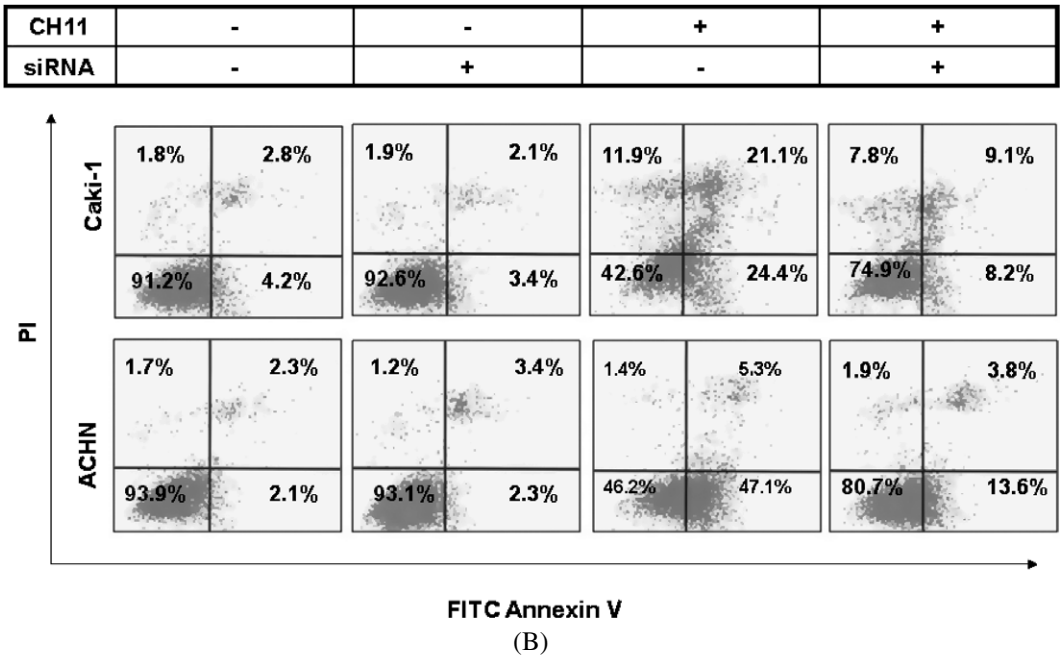
(D)

Fig. 2. (Continued).

vealed that the CH11-induced apoptosis was partially inhibited by either SP600125 or SB-203580. Moreover, the combination of both inhibitors was able to abrogate completely CH11-induced apoptosis, suggesting an important role for both JNK and p38 pathways in the modulation of CH11-induced apoptosis in RCC cells. Next, we examined whether the CH11-induced activation of caspase-8 is associated with CH11-induced JNK and/or p38 pathways. The RCC cells were pretreated with the inhibitors of JNK or p38 pathways before the exposure to CH11 and the activity of caspase-8 was determined by ELISA using IETD-AFC as substrate. Data obtained from ELISA assay (Fig. 3(D)) demonstrated the inhibition of CH11-induced activity of caspase-8 by the inhibitors of JNK or p38 pathways, suggesting the involvement of both

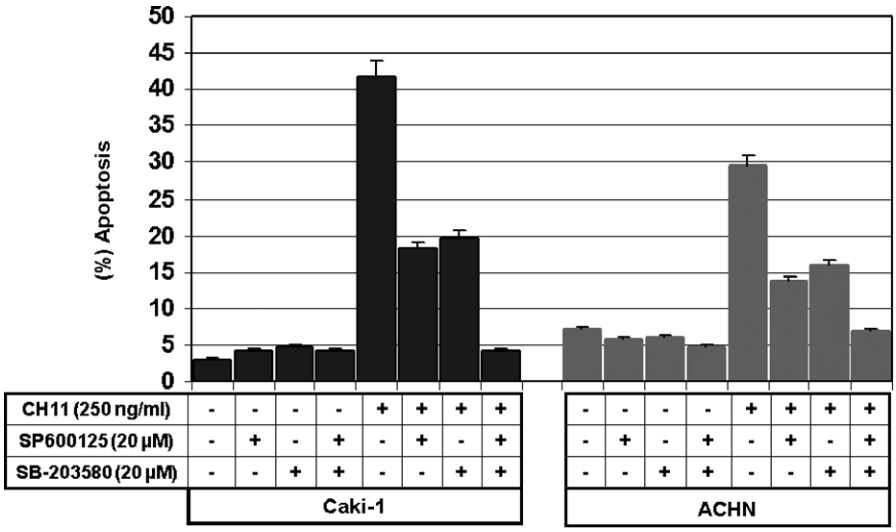


(A)



(B)

Fig. 3. (A) The knockdown of ASK1 by its specific siRNA blocks CH11-induced activation of both JNK and p38. ASK1 expression was monitored by Western blot, whereas, JNK and p38 activity was analyzed by *in vitro* kinase assay. (B) Flow cytometry analysis using annexin V/PI demonstrating the inhibition of CH11-induced apoptosis by ASK1-specific siRNA. Data are representative of three independent experiments. (C) Flow cytometry analysis using annexin V/PI demonstrating the inhibition of CH11-induced apoptosis in RCC cells by the inhibitors of JNK (SP600125), p38 (SB203580) pathways or by caspases inhibitor. Data presented are the mean ± SD of three independent experiments. (D) The activity of caspases-3, 8 and 9 was determined in RCC cells before and after the treatment with CH11 in the presence and the absence of the inhibitors of JNK or p38 using the fluorogenic substrates DEVD-AMC, IETD-AFC and LEHD-AMC, respectively. Data are the mean ± SD of three separate experiments. (E) Western blot analysis demonstrating the inhibition of CH11-induced phosphorylation of FADD and cleavage of Bid by the inhibitors of JNK and p38 pathways in ACHN cells. Total FADD was used as control for FADD phosphorylation, whereas β-actin was used as internal control for loading and transfer. Data are representative of three independent experiments performed separately. (F) Flow cytometry analysis using annexin V/PI demonstrating the abrogation of CH11-induced apoptosis in RCC cells by Ac-IETD-CHO, the inhibitor of caspase-8 (C8 inh.). Data presented are the mean ± SD of three independent experiments.



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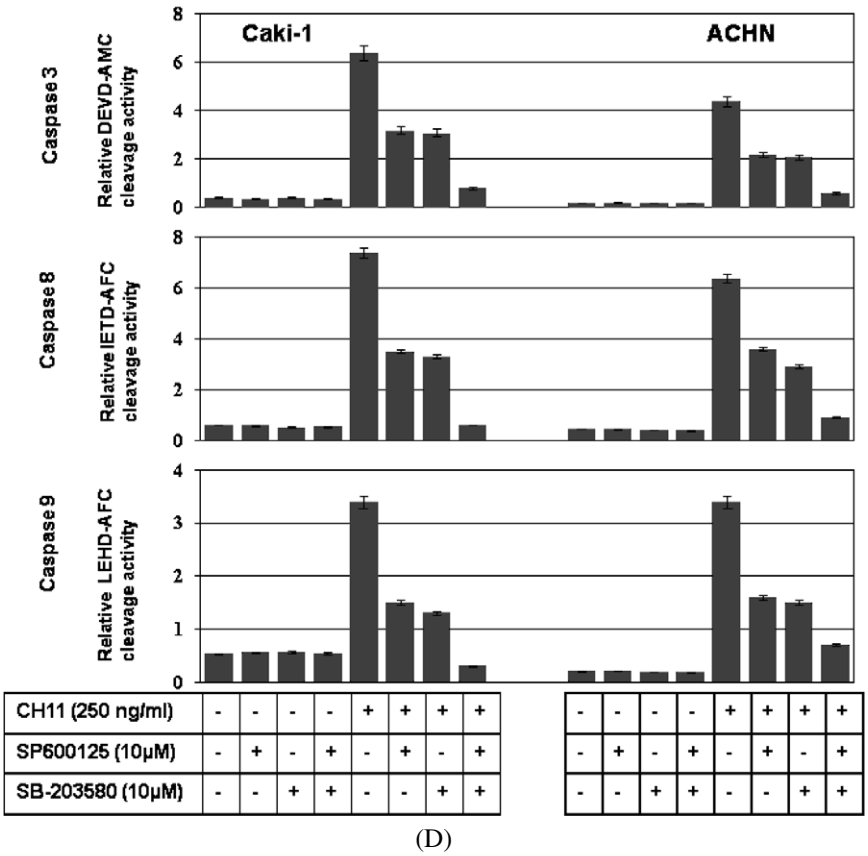
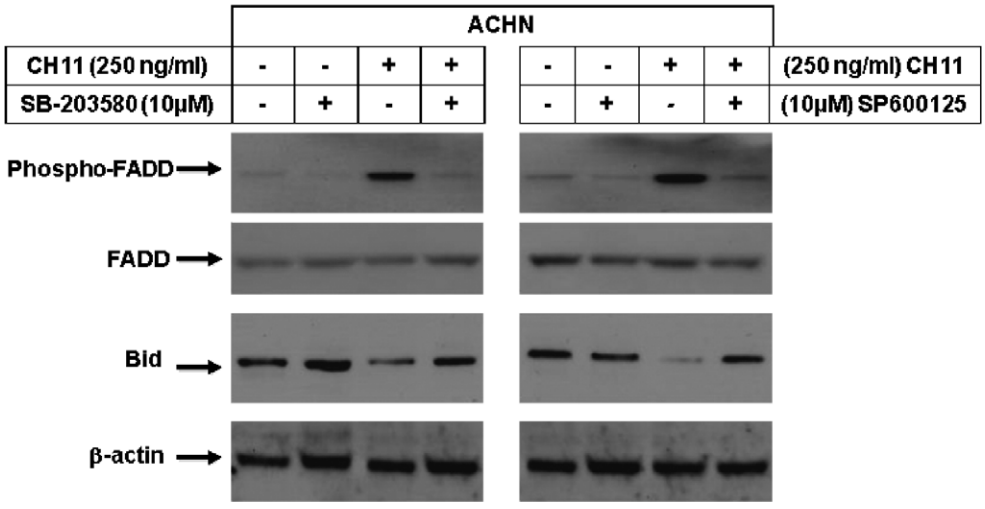


(D)

Fig. 3. (Continued).

pathways in the modulation of CH11-induced activity of caspase-8. In addition, we also showed the inhibition of CH11-induced activation of caspases-9 and 3 by the inhibitors of JNK and p38 (Fig. 3(D)). We further demonstrated the inhibition of CH11-induced phosphorylation of FADD and the cleavage of Bid by



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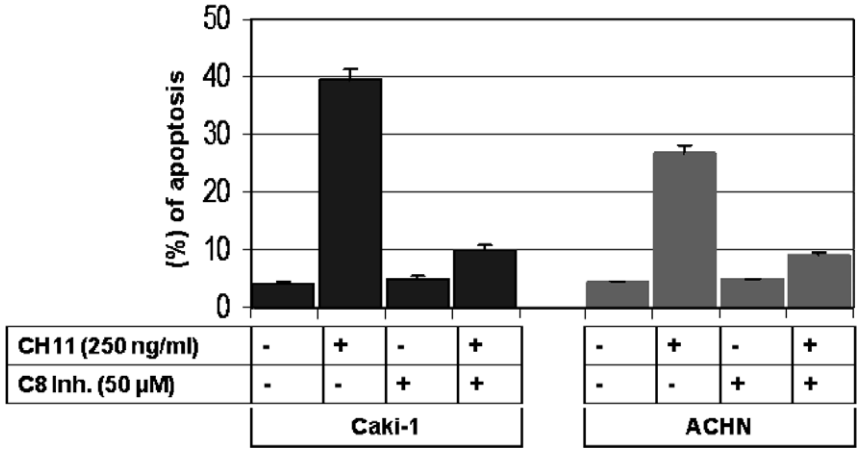

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Fig. 3. (Continued).

the inhibitors of JNK and p38 pathways (Fig. 3(E)). In addition, we demonstrated the effect of JNK, p38 and caspase-8 (Ac-IETD-CHO) inhibitors on the extent of CH11-induced apoptosis in RCC cells by flow cytometry analysis using Annexin V/PI. Figure 3(F) demonstrates the inhibition of CH11-induced apoptosis in RCC cells by the treatment with SP600125, SB-203580 or by Ac-IETD-CHO, suggesting the involvement of the JNK, p38 and caspase-8 in the modulation of CH11-induced apoptosis in RCC cells.

8.4. CH11-induced expression of Bax is regulated by ASK1–JNK/p38 pathways

To investigate whether CH11-induced loss of $\Delta\Psi_m$ is also associated with changes at the expression levels of Bax and Bak proteins, both Caki-1 and ACHN cells were treated with CH11. 48 h later, cells were subjected to Western blot analysis using antibodies specific to Bax and Bak proteins. We found that Bax, but not Bak, is up-regulated at the protein levels in response to the treatment with CH11 (Fig. 4(A)). To examine whether CH11-induced activation of ASK1–JNK/p38 pathways is involved in the regulation of Bax expression, both Caki-1 and ACHN cells were transfected with ASK1-specific siRNA, or pre-treated with the inhibitors of JNK and/or p38 before the exposure to CH11. 48 h later, the cellular Bax mRNA levels were analysed by qRT-PCR. qRT-PCR analysis demonstrated the inhibition of CH11-induced Bax transcription by the knockdown of ASK1, or by the inhibition of both JNK and p38 pathways (Fig. 4(B and C)). These

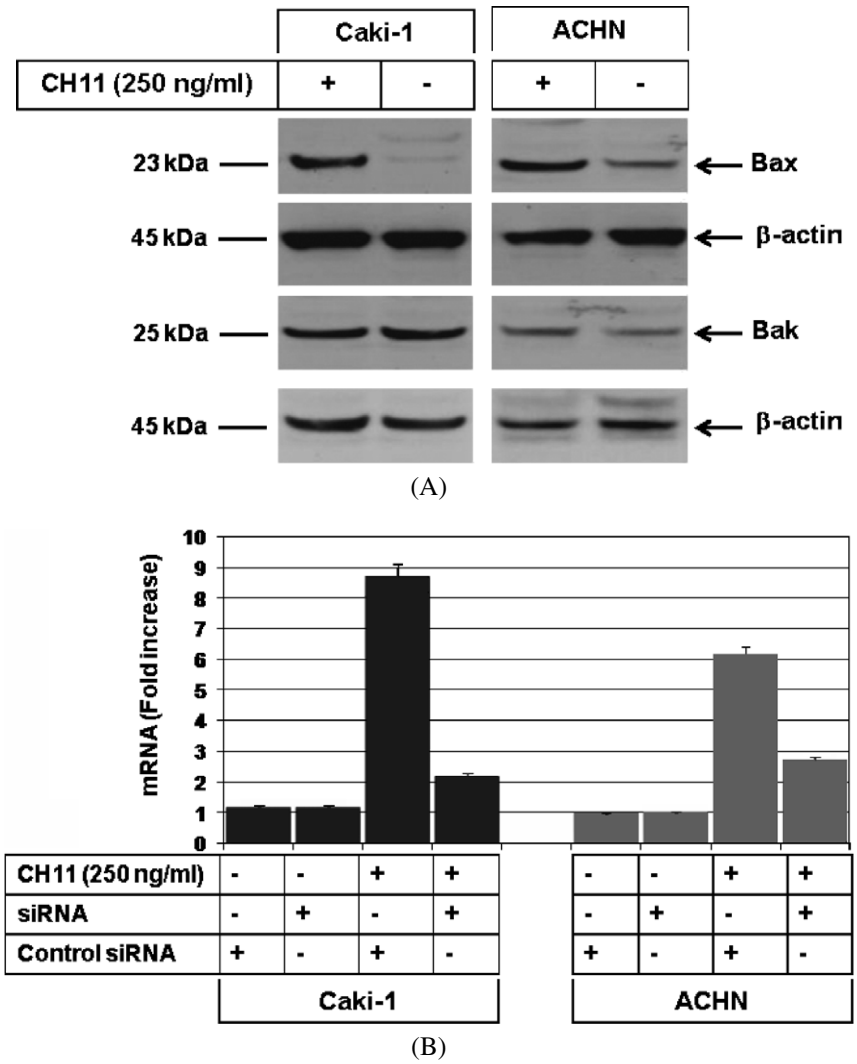


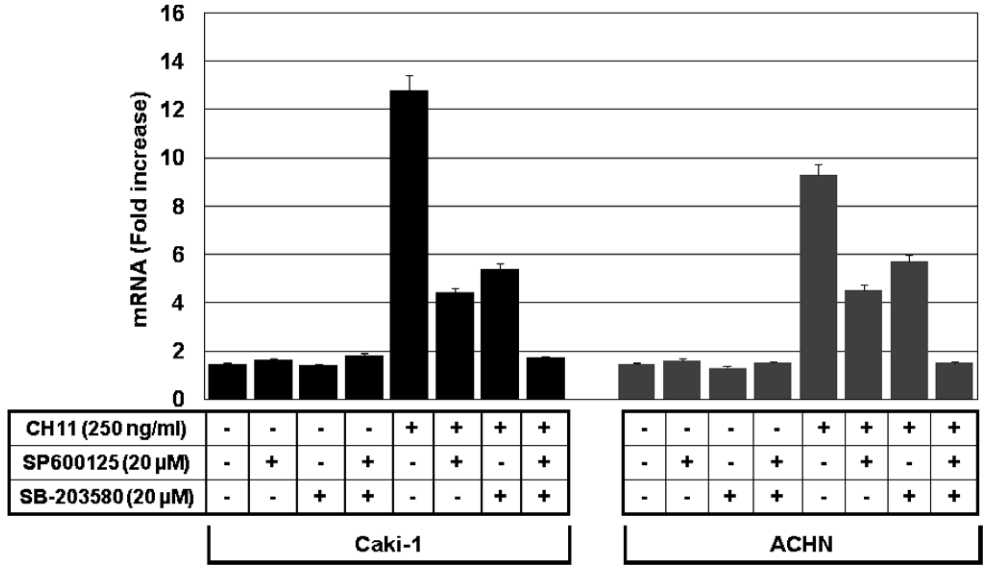
Fig. 4. CH11-induced expression of Bax is regulated by ASK1–JNK/p38 pathways. (A) Western blot analysis of Bax and Bak proteins before and after the treatment with CH11. β-actin was used as internal control for loading and transfer. (B, C) qRT-PCR analysis demonstrating the inhibition of CH11-induced Bax transcription by the knockdown of ASK1 using siRNA or in response to the inhibition of JNK and p38 pathways by their specific inhibitors SP600125 and SB-203580, respectively. Data are representative of three independent experiments.

data suggest the involvement of the ASK–JNK/p38 pathways in the regulation of CH11-induced Bax expression in RCC cells.

8.5. Activation of Bax and Bak proteins is required for the regulation of CH11-induced apoptosis

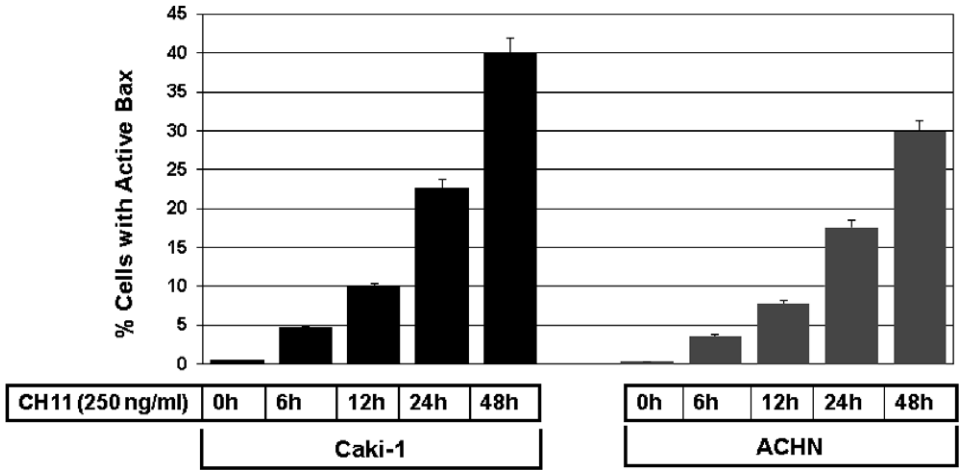
Based on the fact that, the multidomain Bcl-2 family proteins, Bax and Bak, are activated by conformational changes induced by a variety of stimuli [29,30], we analysed activation of these proteins in permeablized cells, using antibodies specific for the activated forms of Bak [30] or Bax [31]. After the exposure to CH11, treated and control cells were subjected to flow cytometry analysis using antibodies specific for activated forms of Bax and Bak proteins. Figure 5(A and B) shows a marked increase in the level of conformationally changed Bax and Bak proteins in both Caki-1 and ACHN cells, which started at 6 h, and increased thereafter up to 48 h, suggesting an important role for Bax and Bak proteins in the modulation of CH11-induced apoptosis in RCC cells.

To evaluate the role of ASK1–JNK/p38 pathways as well as caspase-8 in the regulation of CH11-induced



(C)

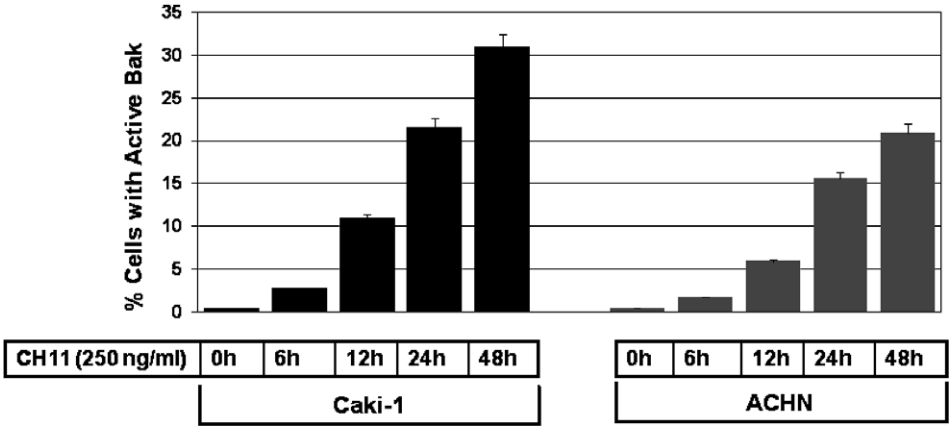
Fig. 4. (Continued).



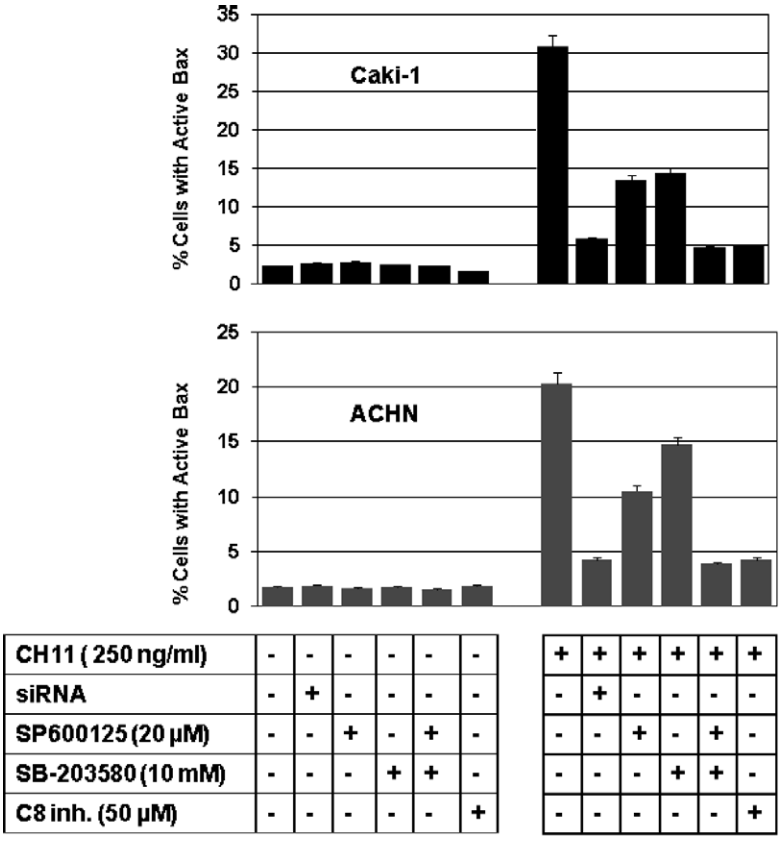
(A)

Fig. 5. (A, B) CH11 induced Bax and Bak activation in RCC cells. Activation of both Bax and Bak increased with time of exposure to CH11. ACHN and Caki-1 cells were grown overnight and then the cells were incubated without CH11 or with CH11 (250 ng/ml) for indicated time. Intracellular flow cytometry was performed using conformation-specific, anti-Bax antibody or anti-Bak. The secondary antibody alone was used as negative control. The percentages of cells with activated Bax (A) or activated Bak (B) are shown. The data are the mean ± SD of three independent experiments. (C) Bax and Bak are required for the modulation of CH11-induced apoptosis. Inhibition of CH11-induced Bax and Bak activation by ASK1-specific siRNA, SP600125, SB-203580, and by Ac-IETD-CHO, the specific inhibitor of caspase-8 (C8 inh.) in both ACHN and Caki-1 cells. (D) Flow cytometry analysis using Bax or Bak specific antibodies demonstrating the conformational changes of Bax and Bak proteins in RCC cell following the treatment with CH11. Data are mean ± SD of three independent experiments. (E) Flow cytometry analysis of mouse embryonic fibroblasts (MEFs), Wilde type (W. type), MEF Bax$^{-/-}$, MEF Bak$^{-/-}$ and MEF Bax$^{-/-}$Bak$^{-/-}$ before and after the treatment with CH11 for 24 h. Results presented are mean ± SD of three independent experiments.

conformational changes of Bax and Bak proteins in RCC cells, both Caki-1 and ACHN cells were transfected with ASK1-specific siRNA or pre-treated with the inhibitors of JNK (SP600125), p38 (SB-203580) or with those of caspase-8 (Ac-IETD-CHO) before the exposure to CH11 for 48 h. Treated and control cells



(B)

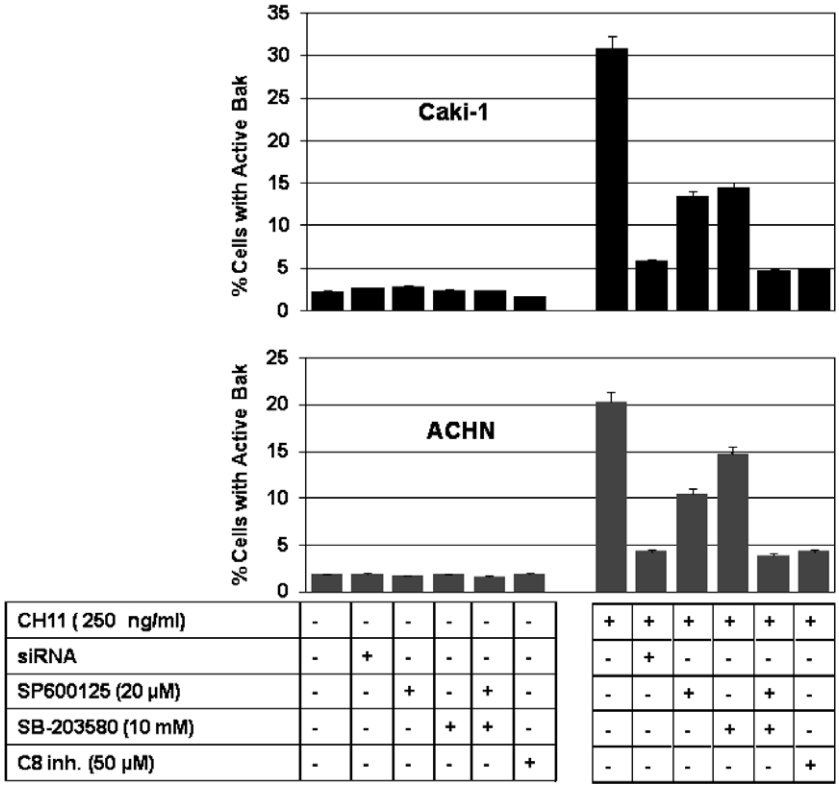


(C)

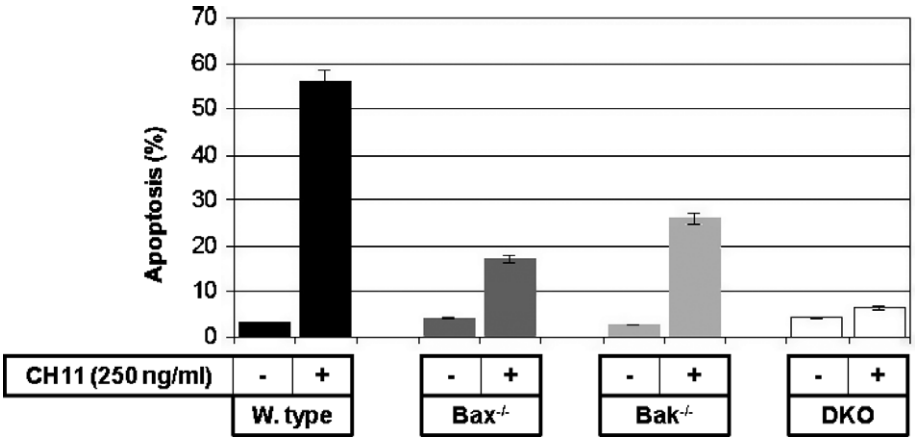
Fig. 5. (Continued).

were subjected to flow cytometry analysis for the measurement of the conformational changes in Bax and Bak proteins. Interestingly, data obtained from flow cytometry analysis (Fig. 5(C and D)) demonstrated the inhibition of CH11-induced activation of Bax and Bak proteins by the ASK1-specific siRNA, as well as by the inhibitors JNK, p38 and caspase-8, suggesting the involvement of ASK1–JNK/p38 pathways and caspase-8 in the regulation of CH11-induced activation of Bax and Bak proteins in RCC cells.

To evaluate the role of Bax and Bak proteins in the modulation of CH11-induced apoptosis, we compared



(D)



(E)

Fig. 5. (Continued).

the sensitivities of Bax and/or Bak deficient mouse embryonic fibroblasts (MEFs) towards CH11-induced apoptosis. The wild-type MEFs were included in the analysis for direct comparison. Apoptosis induction by CH11 was assessed by flow cytometry analysis using Annexin V/PI. Figure 5(E) shows that the exposure of wild-type MEFs to CH11 for 24 h causes a >50-fold increase in percentage of apoptosis when compared with control cells. On the other hand, CH11-induced apoptosis was observed to a significantly lower extent in $Bax^{-/-}$ and $Bak^{-/-}$ MEFs compared to wild-type MEFs. As expected, combined knockout of Bax and Bak offered even greater protection against CH11-induced apoptosis than the knockdown of either Bax

or Bak (Fig. 5(E)). These data provide evidence for the involvement of both Bax and Bak proteins in the modulation CH11-induced apoptosis.

9. Discussion

The present study demonstrates the ability of anti-Fas monoclonal antibody (mAb), CH11 to trigger apoptosis of renal cell carcinoma (RCC) cells and provides an insight into the molecular mechanisms, which are involved in the regulation of this event. In addition, our study shows an essential role for ASK1, and both JNK and p38 pathways in the modulation of CH11-induced apoptosis and confirms the validity of Fas activation as a potential therapeutic approach for the treatment of RCC.

Based on the current study, Fig. 6 demonstrates a proposed model for Fas-induced pathways to apoptosis in RCC cells. Upon the ligation of anti-Fas antibody (CH11) to FasR, the ASK1–JNK/p38 signalling pathways become activated, leading to phosphorylation of FADD, activation of caspase-8 and cleavage of Bid as well as the enhancement of the DNA-binding activity of the transcription factors AP-1 and ATF-2, that leads to the induction of Bax mRNA and protein expression. In the cytoplasm of CH11-treated cells, Bax and Bak proteins become more active via their conformational changes that are mediated by truncated Bid (tBid). The accumulation of both Bax and Bak on the mitochondria leads to the loss of mitochondrial membrane potential that is characterized by cytochrome c release, cleavage of caspases-9 and 3, and PARP.

As widely reported, Fas can activate two distinct signaling pathways. One of these pathways involves FADD, which recruits procaspase-8 and activates protease cascade leading to apoptosis [32–34], whereas, the other pathway is mediated by Daxx via a mechanism including the activation of apoptosis signal-regulating kinase 1 (ASK1) and its downstream signal-

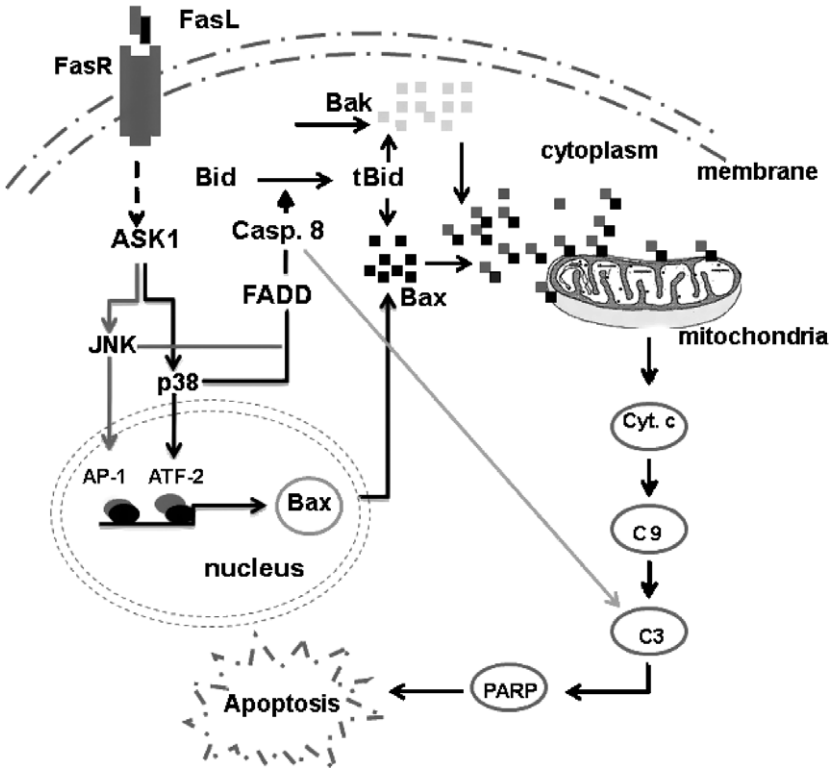


Fig. 6. Model for Fas-induced pathways to apoptosis in RCC cells. Upon the ligation of anti-Fas antibody (CH11) to FasR, the ASK1–JNK/p38 signaling pathways become activated, leading to phosphorylation of FADD, activation of caspase-8 and cleavage Bid as well as the enhancement of the DNA-binding activity of the transcription factors AP-1 and ATF-2 leading to the induction of Bax expression. In the cytoplasm of CH11-treated cells, Bax and Bak proteins become more active via their conformational changes that are mediated by truncated Bid (tBid). The accumulation of both Bax and Bak on the mitochondria leads to the loss of mitochondrial ($\Delta\Psi_m$) membrane potential that is characterized by cytochrome c release, cleavage of caspases-9 and 3, and PARP.

ing pathways including JNK and p38 [35,36], that in turn mediates the phosphorylation of FADD, thereby leading to the activation of caspase-8 [37].

The activation of JNK [38] and/or p38 [39] has been reported to trigger apoptotic pathways initiated by Fas and mitochondria. In the present study, CH11-induced apoptosis of RCC cells was found to be mediated by ASK1–JNK/p38 pathways through the phosphorylation of FADD and subsequent caspase-8 activation that in turn mediates the cleavage of Bid. This conclusion is supported by our findings demonstrating that the blocking of ASK1, JNK, or p38 resulted not only in the inhibition of apoptosis, but also in the abrogation of CH11-induced FADD phosphorylation, caspase-8 activation, Bid cleavage, and activation of both Bax and Bak proteins. Simultaneously blocking caspase-8 activation totally abolished CH11-induced Bid cleavage, Bax and Bak activation and the subsequent apoptosis. These data, therefore, clearly suggest the role of ASK1–JNK/p38 pathway as mediator of CH11-induced FADD-caspase-8-Bid pathway leading to the translocation of both Bax and Bak proteins to mitochondria and subsequent mitochondrial dysregulation, which is characterized by the loss of $\Delta\Psi_m$, cytochrome c release, cleavage of caspase-9, caspase-3 and PARP [22]. The activated Caspase-8 propagates the apoptotic signal by activating downstream caspases through proteolytic cleavage [40], as well as the enhancement of the pro-apoptotic potential of Bid by removing the inhibitory N-terminal domain, allowing truncated Bid (tBid) to interact with its receptor on the mitochondria [41], leading to accumulation and subsequently the translocation of both Bax and Bak proteins to mitochondria and thereby causing the loss of $\Delta\Psi_m$ that in turn activate proteolytic cascade [22]. Consistent with this notion, our data demonstrate the ability of ASK1 inhibition to block Fas-induced apoptosis as well as the activation of JNK and p38 pathways, phosphorylation of FADD, and the activation of caspase-8 and subsequently the cleavage of Bid, suggesting a central role for ASK1 in the modulation of Fas-induced pathway to apoptosis in RCC cells via Daxx-dependent mechanism. Moreover, the protection of RCC cells from CH11-induced apoptosis by inhibition of JNK and p38 pathways, further supports a critical role for ASK1 kinase in JNK and p38 activation, and apoptosis induced. Thus, our findings suggest a Fas-Daxx-ASK1 axis in activating JNK and p38 MAP kinase cascades by Fas.

Indeed, Bid BH3-induced Bax activation may be a catalytic process, in which activated Bax propagates its pro-apoptotic activity by subsequent Bax BH3-mediated auto-activation as described [35,42]. Bax and Bak constitute the gateway to mitochondria-dependent apoptosis [31]. When activated, Bax and Bak oligomerize and this is associated with the permeabilization of the outer mitochondrial membrane leading to the release of apoptotic factors such as cytochrome c. This event, in turn, results in the activation of the caspase cascade that functionally mediates cellular demolition.

In the present study, we noted that the upregulation of Bax at both mRNA and protein levels as well as the activation of both Bax and Bak in RCC cells by the treatment with CH11. In addition, the inhibition of Fas-induced activation of both Bax and Bak by the knockdown of ASK1, by the inhibition JNK and p38 pathways, or by the inhibitor of caspase-8 (Ac-IETD-CHO), confirmed the involvement of both ASK1–JNK/p38 pathways and caspase-8 in the regulation of Fas-induced activation and thereby the translocation of both Bax and Bak proteins to mitochondria. Moreover, the inhibition of Fas-induced apoptosis in $Bax^{-/-}/Bak^{-/-}$ MEFs suggested an important role for both Bax and Bak proteins in the modulation of Fas-mediated pathways to apoptosis.

In summary, our data provide insight into the molecular mechanism of CH11-induced pathways to apoptosis in RCC cells and demonstrate that the activation of Fas-signalling pathways to apoptosis may be an alternative therapeutic approach for the treatment of patients with advanced RCC.

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