

## Anti-CD146 monoclonal antibody AA98 inhibits angiogenesis via suppression of nuclear factor- $\kappa$ B activation

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### Abstract

Our previous study showed that an anti-CD146 monoclonal antibody (mAb), AA98, which was raised against the vascular endothelial cells stimulated by a conditioned medium from hepatocarcinoma SMMC 7721 cells (SMMC 7721-CM), inhibited cell migration, angiogenesis, and tumor growth. However, the underlying mechanism was not elucidated. The objective of this study was to understand the mechanism by which mAb AA98 inhibits the endothelial cell migration and angiogenesis that is induced by SMMC 7721-CM. Using confocal imaging and biochemical studies, we found that SMMC 7721-CM induced nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation through the upstream p38 mitogen-activated protein kinase pathway, leading to the up-regulation of matrix metalloproteinase 9 and intercellular adhesion molecule 1 expression. Interestingly, all these activities stimulated by SMMC 7721-CM could be effectively inhibited by mAb AA98 in a dose- and time-dependent manner. Our data showed that the engagement of mAb AA98 with membrane protein CD146 inhibited p38 mitogen-activated protein kinase phosphorylation, suppressed NF- $\kappa$ B activation, and down-regulated matrix metalloproteinase 9 and intercellular adhesion molecule 1 expression, suggesting that the suppression of NF- $\kappa$ B is a critical point for the inhibitory function of mAb AA98 on endothelial cell migration, angiogenesis, and tumor metastasis. These results will provide clues for a better understanding of the mechanisms underlying tumor angiogenesis as well as antiangiogenesis therapy. [Mol Cancer Ther 2006;5(11):2872-8]

### Introduction

Cancer metastasis is a complex multistep process involving tumor cell proliferation, migration, invasion, and angiogenesis. An important early event in the development of metastasis is angiogenesis. Most tumor cells secrete a variety of cytokines and proangiogenic molecules, such as fibroblast growth factors, vascular endothelial growth factors (1), IL-8 (2), and extracellular matrix proteinases (3), which promote angiogenesis and metastasis. Although the mechanisms for these factors regulating cell metastasis and angiogenesis are largely undefined, many studies have shown that the pleiotropic transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) can be activated by the cytokines from tumors and that NF- $\kappa$ B plays an important role in the control of tumor metastasis, angiogenesis, and hence, oncogenesis (4).

NF- $\kappa$ B is an inducible transcription factor that belongs to the Rel/NF- $\kappa$ B family. NF- $\kappa$ B has two forms, homodimers and heterodimers, and the heterodimer (p50/p65) is a major NF- $\kappa$ B complex in most cells. These complexes are typically retained in the cytoplasm by inhibitory I $\kappa$ B proteins. Upon stimulation, I $\kappa$ B is rapidly phosphorylated and degraded, permitting NF- $\kappa$ B activation and nuclear translocation. NF- $\kappa$ B activation up-regulates many gene expressions, such as proangiogenic factors and matrix metalloproteinases (MMP) and intercellular adhesion molecule 1 (ICAM-1). MMPs are a family of zinc-dependent endopeptidases (5). Among the MMP family members, MMP-2 and MMP-9 have shown key roles in tumor cell invasion, metastasis, and angiogenesis by degradation of extracellular matrix.

It is well known that cell adhesion molecules such as ICAM-1 could mediate tumor cell migration, angiogenesis, and metastasis (6). CD146, also named MUC18, Mel-CAM, or MCAM, is a newly recognized cell adhesion molecule belonging to the immunoglobulin superfamily (7-9). It was initially identified as a progression marker of melanoma, and plays an important role in promoting melanoma progression and metastasis (7). A fully humanized antibody against CD146, ABX-MA1, has been reported as an inhibitor to block the metastasis of human melanoma and disrupt blood vessel formation *in vitro* (10). CD146 expression can be modulated within the tumor microenvironment. A recent study reported that CD146 expression varied in circulating endothelial cells with increased or different tumor burdens (11). Our study has also shown that CD146 is an inducible molecule on the stimulated endothelial cells using conditioned medium from hepatocarcinoma SMMC 7721, named SMMC 7721-CM. We used SMMC 7721-CM-stimulated endothelial cells as an antigen to immunize mice, and generated monoclonal antibody

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(mAb) AA98, which specifically targets the CD146 molecule. Our animal studies showed that mAb AA98 inhibited angiogenesis and human tumor growth in xenografted mice (12). However, the underlying mechanism was not elucidated.

In this study, we attempt to clarify the mechanism through which mAb AA98 inhibits endothelial cell migration and angiogenesis, which are induced by SMMC 7721-CM. Using imaging and biochemical studies, we found that the engagement of mAb AA98 with membrane protein CD146 could inhibit p38 mitogen-activated protein kinase (MAPK) phosphorylation, suppress NF- $\kappa$ B activation, and down-regulate MMP-9 as well as ICAM-1 expression, suggesting that the suppression of NF- $\kappa$ B is a critical point for the inhibitory function of mAb AA98 on endothelial cell migration, angiogenesis, and tumor metastasis.

## Materials and Methods

### Cell Lines and Culture Conditions

The hepatocarcinoma SMMC 7721 cell line was obtained from American Type Culture Collection (Rockville, MD). Primary human umbilical vein endothelial cells (HUVEC) were prepared from human umbilical cords as previously described (13). All the cells were cultured in DMEM containing 10% FCS.

### Treatment of HUVECs with Stimulator or Inhibitor

The primary HUVECs were stimulated by SMMC 7721-CM for 24 hours in culture. Then, the stimulated cells were treated with inhibitors, either mAb AA98 in different concentrations for different times, or p38 MAPK inhibitor, SB203580 (10 mmol/L) for 30 minutes. Isotype-matched mIgG was used as a control.

### Preparation of Cell Lysates

For the analysis of NF- $\kappa$ B or I $\kappa$ B, cytoplasmic and nuclear extracts were prepared. Briefly, the treated cells were washed twice and scraped into 1.0 mL of ice-cold PBS. After centrifugation at 3,000  $\times$  g at 4°C for 5 minutes, the cell pellet was lysed in 60  $\mu$ L of lysis buffer A [10 mmol/L Tris (pH 8.0), 1.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L 1,4-DTT, 0.1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, and 25  $\mu$ g/mL aprotinin] and then incubated on ice for 15 minutes followed by centrifugation at 12,000  $\times$  g at 4°C for 15 minutes. The supernatants (cytoplasmic fraction) were saved in a tube and the pellets containing nuclei were suspended in 60  $\mu$ L of buffer B [10 mmol/L Tris (pH 8.0), 50 mmol/L KCl, 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, and 25  $\mu$ g/mL aprotinin] at 4°C for 30 minutes. After centrifugation at 12,000  $\times$  g at 4°C for 15 minutes, the supernatants were saved as nuclear extracts.

In order to analyze the phosphorylation of extracellular signal-regulated kinase (ERK), p38 MAPK and ICAM-1, the treated cells were lysed in lysis buffer C [150 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris (pH 8.0), 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 25  $\mu$ g/mL aprotinin, 1 mmol/L sodium vanadate, and

50 mmol/L sodium fluoride] for 20 minutes on ice. Lysates were centrifuged at 10,000  $\times$  g at 4°C for 5 minutes and the supernatants were collected for Western blot assay.

### Western Blot

Samples (26  $\mu$ g from the cytoplasmic fraction and 19  $\mu$ g from the nuclear extracts) were first separated by 10% SDS-PAGE and then transferred to a Hybond membrane (Amersham, Piscataway, NJ). The membranes were blocked with 5% milk in PBS for 1 hour, incubated for 2 hours with primary antibodies, either anti-NF- $\kappa$ B (p65), anti-I $\kappa$ B $\alpha$ , anti- $\beta$ -actin, or anti-phosphorylated p38, anti-phosphorylated ERK1/2, and anti-ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and then probed for 1 hour with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Pierce, Rockford, IL). After extensive washing with PBST, the target proteins were detected on the membranes by enhanced chemiluminescence (Pierce).

### Confocal Microscopy

HUVECs were plated on coverslips and cultured in a six-well plate. After stimulation for 24 hours with SMMC 7721-CM, followed by treatment with 50  $\mu$ g/mL of either mAb AA98 or control mIgG for 1 hour, the cells were washed with PBS, fixed in 3% cold formaldehyde in PBS for 4 minutes, and then permeabilized with 0.2% Triton X-100. After washing with PBS, the cells were blocked in 5% normal goat serum for 30 minutes, and then incubated with anti-NF- $\kappa$ B (p65 or p50) antibody (Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with FITC-conjugated anti-mouse IgG (The Jackson Laboratory, Bar Harbor, ME) and Cy3-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) for 1 hour at room temperature. Finally, the coverslips were examined with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

### Zymography

The activities of MMP-2 and MMP-9 were determined using substrate-impregnated gels (14) with minor modifications. HUVECs were plated in a 24-well plate, stimulated using SMMC 7721-CM for 24 hours, and then treated with 50  $\mu$ g/mL of mAb AA98 or control mIgG for 24 hours. After each treatment above, the culture medium was removed and replaced with serum-free medium for continuous culture overnight. The culture supernatants were collected and an equal amount of protein (0.92  $\mu$ g) was analyzed using a gelatin-impregnated (1 mg/mL) 10% SDS-PAGE, followed by 30 minutes of shaking in 2.5% Triton X-100 in 50 mmol/L of Tris (pH 7.6) twice. The gels were then incubated for 18 hours at 37°C in 50 mmol/L Tris, 0.2 mol/L NaCl, 5 mmol/L CaCl<sub>2</sub>, and 1% Triton X-100 (pH 7.6). Finally, the gels were stained with 0.5% Coomassie blue G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H<sub>2</sub>O. The gelatinolysis in the gels was visualized as transparent bands.

### RNA Isolation and Reverse Transcription-PCR Analysis

HUVECs were first stimulated for 24 hours with SMMC 7721-CM, and then treated with 50  $\mu$ g/mL of either mAb AA98 or control mIgG for 24 hours. Total RNA was isolated using Trizol reagent (Invitrogen, San Diego, CA) from the



treated cells. Aliquots of 1  $\mu$ g of total RNA were used for the first-strand cDNA synthesis in 20  $\mu$ L of the reaction volume using 100 units of Superscript II reverse transcriptase (Invitrogen). The primers used for human MMP-9 amplification were: 5'-GAGACCGGTGAGCTGGA-TAG-3' (sense) and 5'-GTACACGCGAGTGAAGGTGA-3' (antisense). The primers used for ICAM-1 were: 5'-CGT-GCCGCACTGAACTGGAC-3' (sense) and 5'-CCTCA-CACTTCACTGTCACCT-3' (antisense). The primers used for glyceraldehyde-3-phosphate dehydrogenase were: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (sense) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (antisense). PCR amplifications were run for 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and 72°C for 7 minutes; then held at 4°C. PCR products were analyzed using electrophoresis in 1% agarose gels followed by staining with ethidium bromide.

## Results

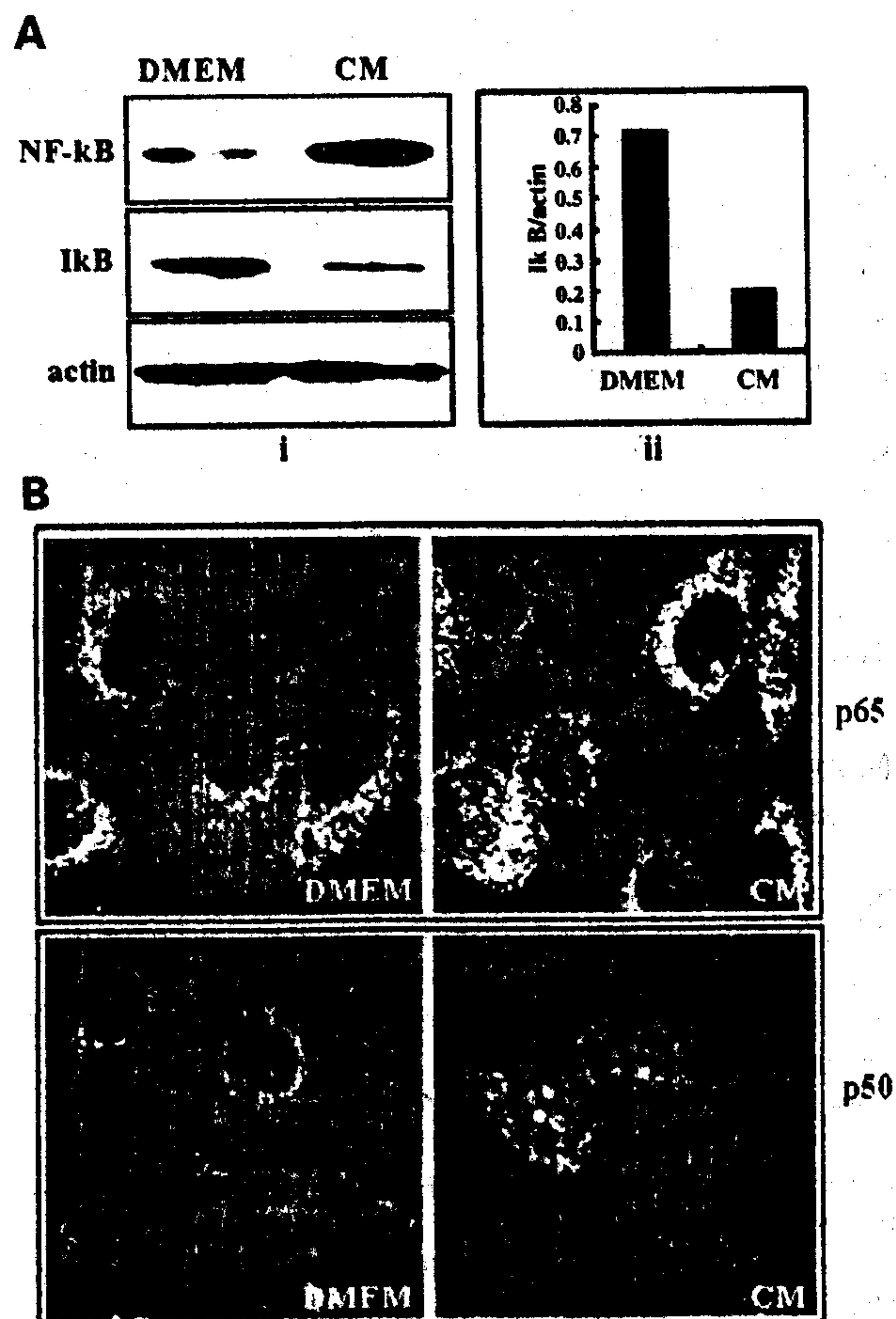
### SMMC 7721-CM Activated NF- $\kappa$ B in HUVECs

In our previous study, we found that SMMC 7721-CM promoted the proliferation and migration of vascular endothelial cells, and that mAb AA98 inhibited all these activities. Given the fact that many tumor cells secrete a variety of proangiogenic molecules, which promote angiogenesis and metastasis through NF- $\kappa$ B activation, we hypothesize that the activation of endothelial cells stimulated by SMMC 7721-CM is due to NF- $\kappa$ B activation. To test this hypothesis, we first analyzed NF- $\kappa$ B and I $\kappa$ B $\alpha$  activation in HUVECs before and after treatment with SMMC 7721-CM. Western blot assays show significantly increased levels of NF- $\kappa$ B (p65) in the nuclear fraction after the cells were treated with SMMC 7721-CM. Conversely, I $\kappa$ B $\alpha$  levels in the cytosol fraction dramatically decreased, as compared with the fractions of HUVECs cultured in normal DMEM (Fig. 1A).

To confirm these results, we used a confocal light microscope to directly observe the nuclear translocation of NF- $\kappa$ B in the stimulated cells. The confocal images showed that little or no NF- $\kappa$ B was observed in nuclei when the cells were cultured in normal DMEM. However, after stimulation with SMMC 7721-CM, NF- $\kappa$ B (p65 and p50) was activated and translocated into the nuclei, resulting in a concentration of FITC-labeled NF- $\kappa$ B (p65) and Cy3-labeled NF- $\kappa$ B (p50) presented in the nuclei (Fig. 1B). These results strongly indicate that I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation are induced by SMMC 7721-CM.

### mAb AA98 Inhibits NF- $\kappa$ B Activation Induced by SMMC 7721-CM

Interestingly, we found that the NF- $\kappa$ B (p50 and p65) nuclear translocation induced by SMMC 7721-CM was efficiently inhibited by mAb AA98, which has been identified as an inhibitor of angiogenesis (12), whereas an isotype-matched mIgG has no effect (Fig. 2A). Moreover, the inhibitory effect of AA98 on NF- $\kappa$ B activation was found to be both dose- and time-dependent. A decreasing



**Figure 1.** SMMC 7721-CM induces NF- $\kappa$ B nuclear translocation and I $\kappa$ B $\alpha$  degradation in HUVECs. **A, i,** Western blot shows the changes of NF- $\kappa$ B p65 in the nuclear fraction and I $\kappa$ B $\alpha$  in the cytosol fraction after HUVECs were cultured with SMMC 7721-CM (CM) or normal medium (DMEM).  $\beta$ -Actin was used as an internal control for I $\kappa$ B $\alpha$  in cytosol. **ii,** quantification of the amount of I $\kappa$ B $\alpha$  in the bands on Western blot using Quantity One software (Bio-Rad). **B,** confocal imaging of the translocation of NF- $\kappa$ B (p65 and p50) into the nuclei after HUVECs were treated with CM and DMEM.

level of NF- $\kappa$ B (p65) was detected in the nuclear fraction both after the cells were treated with an increasing amount of mAb AA98 (0, 10, 30, or 50  $\mu$ g/mL) and also with increasing time (0, 30, 60, or 90 minutes). In contrast, I $\kappa$ B $\alpha$  in the cytosol fraction was increased in the same mAb AA98-treated cells (Fig. 2B and C). However, the control mIgG did not show any effect on either NF- $\kappa$ B or I $\kappa$ B $\alpha$  activation, even at the highest concentration (50  $\mu$ g/mL) and longest time (90 minutes). These results show that SMMC 7721-CM-induced NF- $\kappa$ B activation and nuclear translocation was specifically abolished by mAb AA98.

### mAb AA98 Did Not Inhibit NF- $\kappa$ B Activation Induced by Interleukin 1 $\beta$ or Tumor Necrosis Factor $\alpha$

In order to find which factor in SMMC 7721-CM is a major contributor to activating NF- $\kappa$ B through the



CD146-induced signal pathway, we used interleukin 1 $\beta$  (IL-1 $\beta$ ) or tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) to treat HUVECs for 30 minutes, and then added mAb AA98 in the culture for another 1 hour. The results showed that both factors, IL-1 $\beta$  and TNF- $\alpha$ , induced I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation. However, this activation was not inhibited by mAb AA98 (Fig. 3A and B), indicating that the unidentified NF- $\kappa$ B inducer may trigger a pathway which is different from IL-1 $\beta$  and TNF- $\alpha$ .

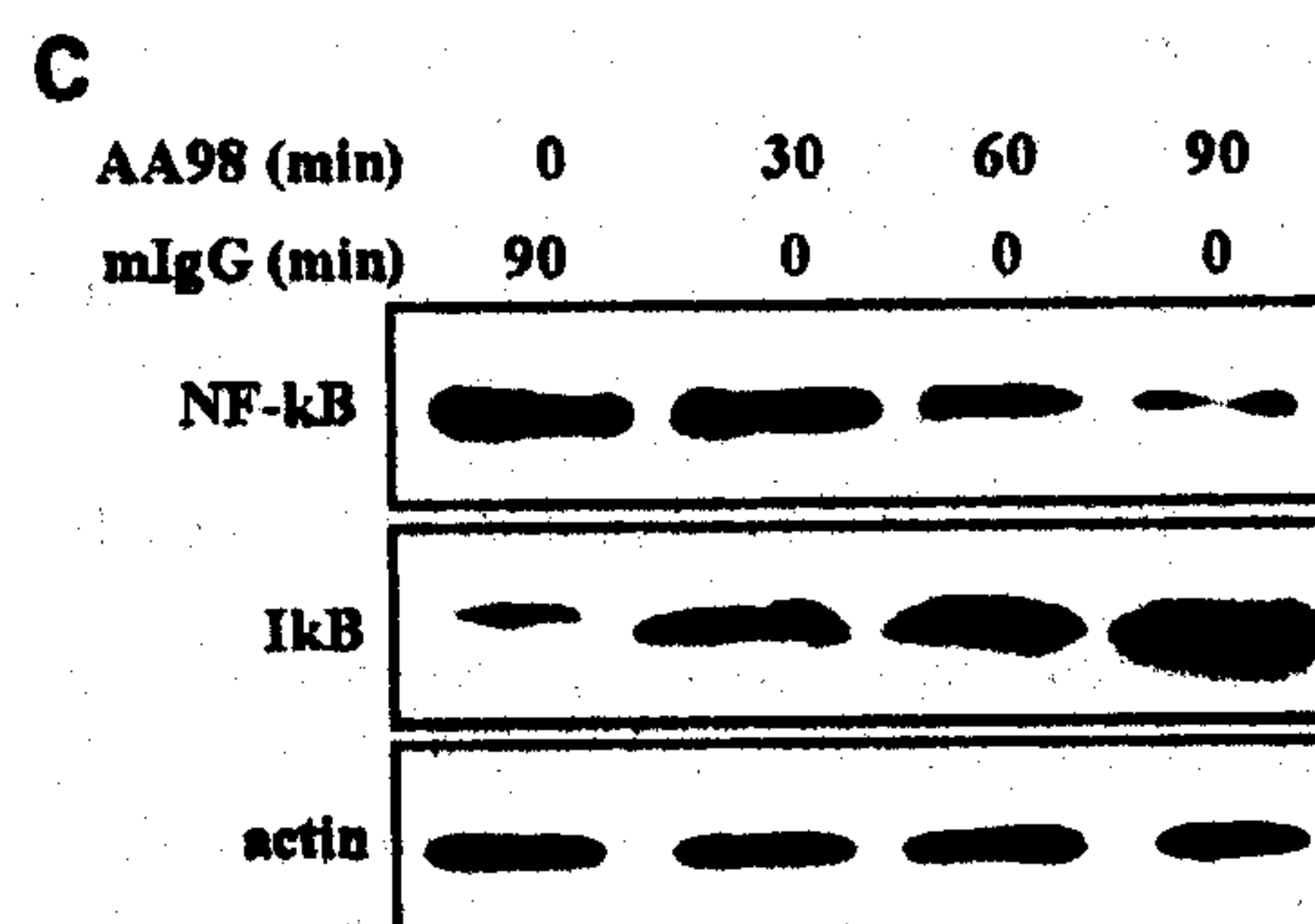
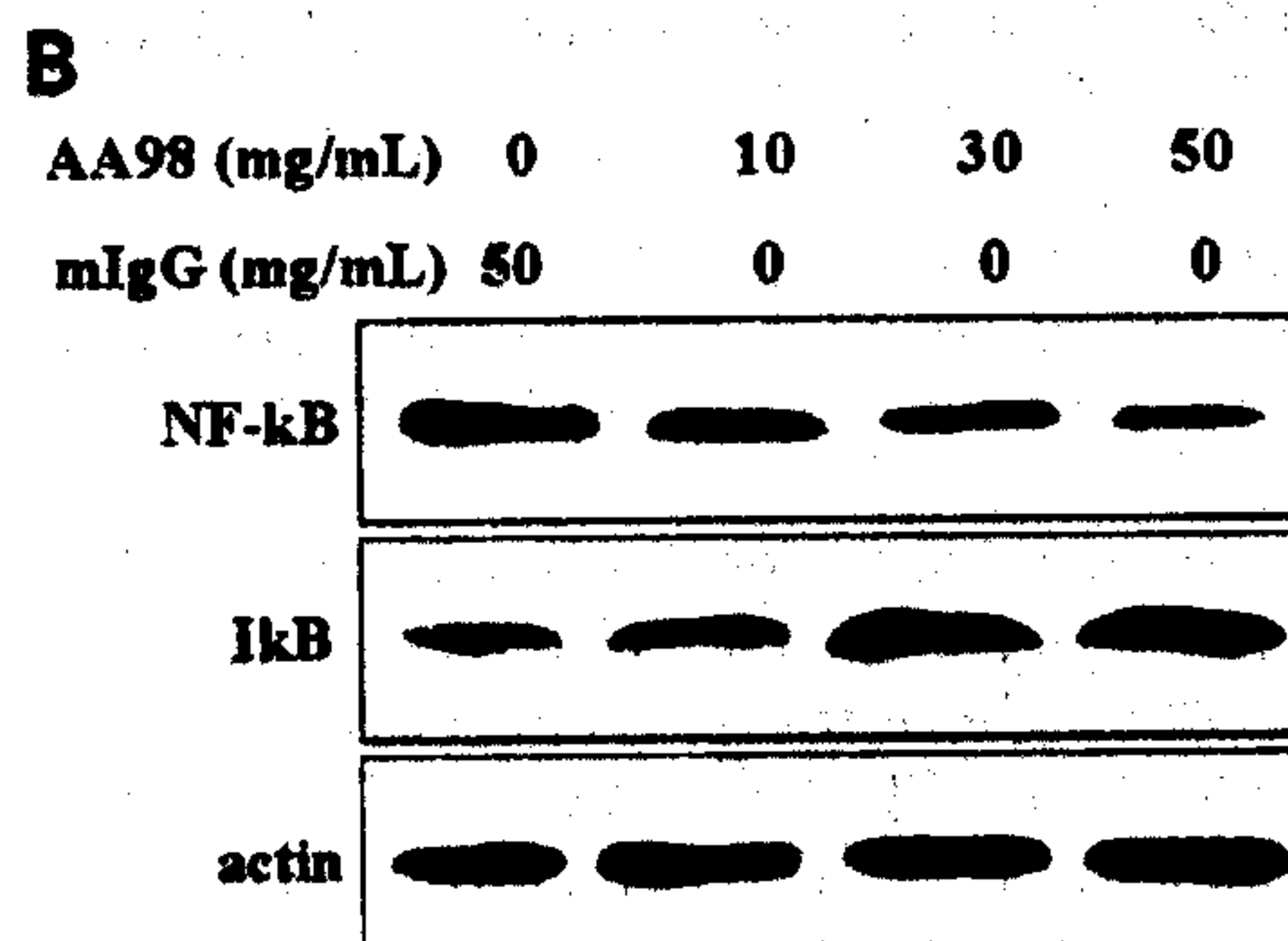
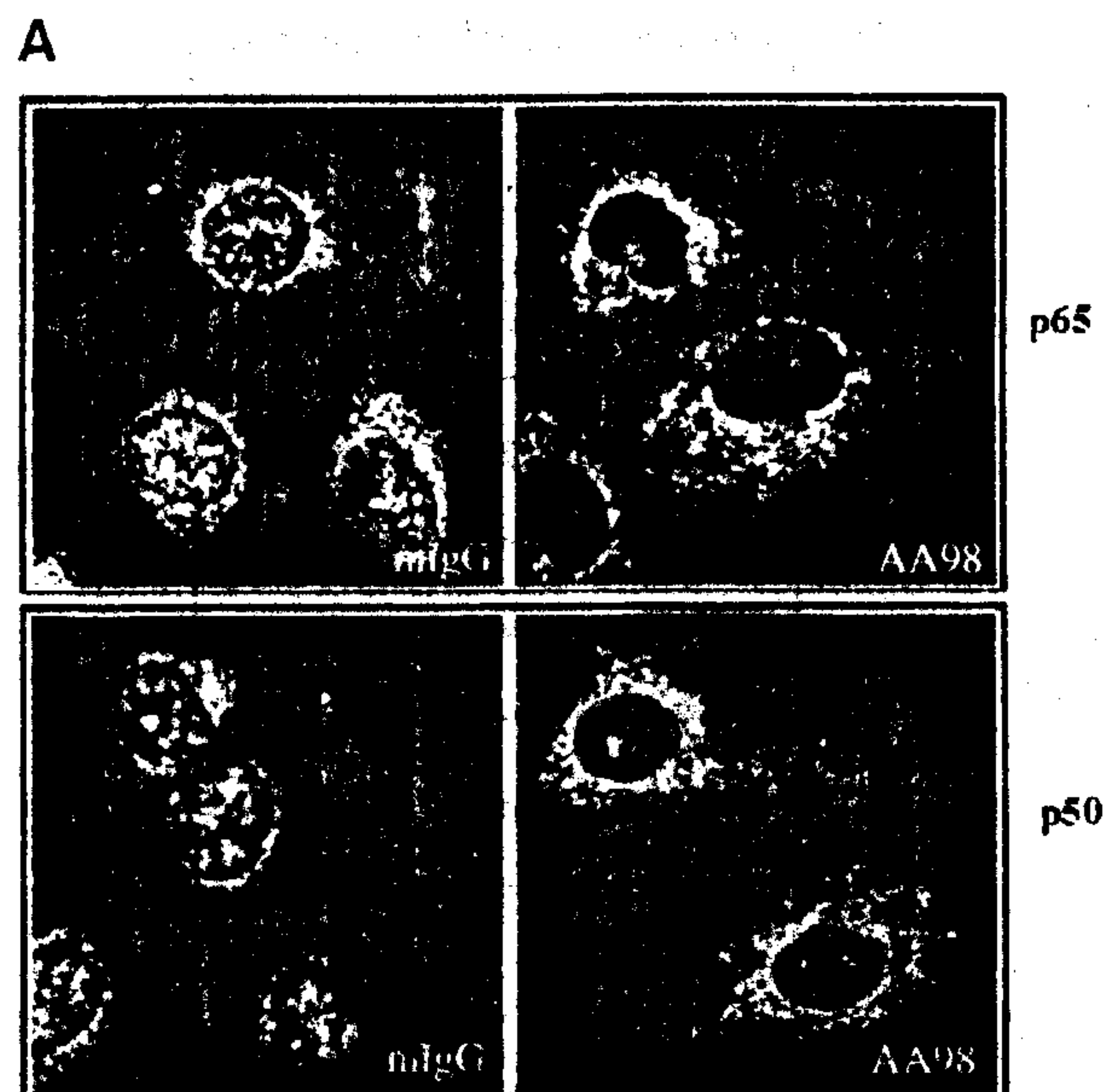
#### mAb AA98 Inhibits NF- $\kappa$ B Activation via the Suppression of p38 MAPK

It has been reported that NF- $\kappa$ B can be activated by multiple factors through the MAPK pathway, which plays an important role in transducing extracellular signals into cellular responses. Two members of the MAPK family, ERK and p38 MAPK, are involved in the activation of NF- $\kappa$ B (15–17). We next investigated whether the SMMC 7721-CM-induced NF- $\kappa$ B activation was via the ERK and/or p38 MAPK pathway, and whether this activation was inhibited by mAb AA98 or SB203580, a specific inhibitor of p38 MAPK. To address this issue, HUVECs were first stimulated with SMMC 7721-CM and then treated with either mAb AA98 or SB203580. Western blot analysis showed the phosphorylation of ERK and p38 MAPK in the treated cells. The results indicated that SMMC 7721-CM specifically induced p38 MAPK phosphorylation, but not ERK, and this activated p38 MAPK could be selectively inhibited by mAb AA98 (Fig. 4A and B). Moreover, we found that I $\kappa$ B $\alpha$  was remarkably reduced and NF- $\kappa$ B (p65) was obviously increased after the cells treated with SMMC 7721-CM, and the p38 inhibitor, SB203580, could inhibit I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation in the treated cells (Fig. 4C). These observations indicate that the p38 MAPK was an upstream pathway of the NF- $\kappa$ B activation induced by SMMC 7721-CM in the stimulated HUVECs, and that mAb AA98 inhibited NF- $\kappa$ B activation via the suppression of the p38 MAPK but not the ERK pathway in HUVECs.

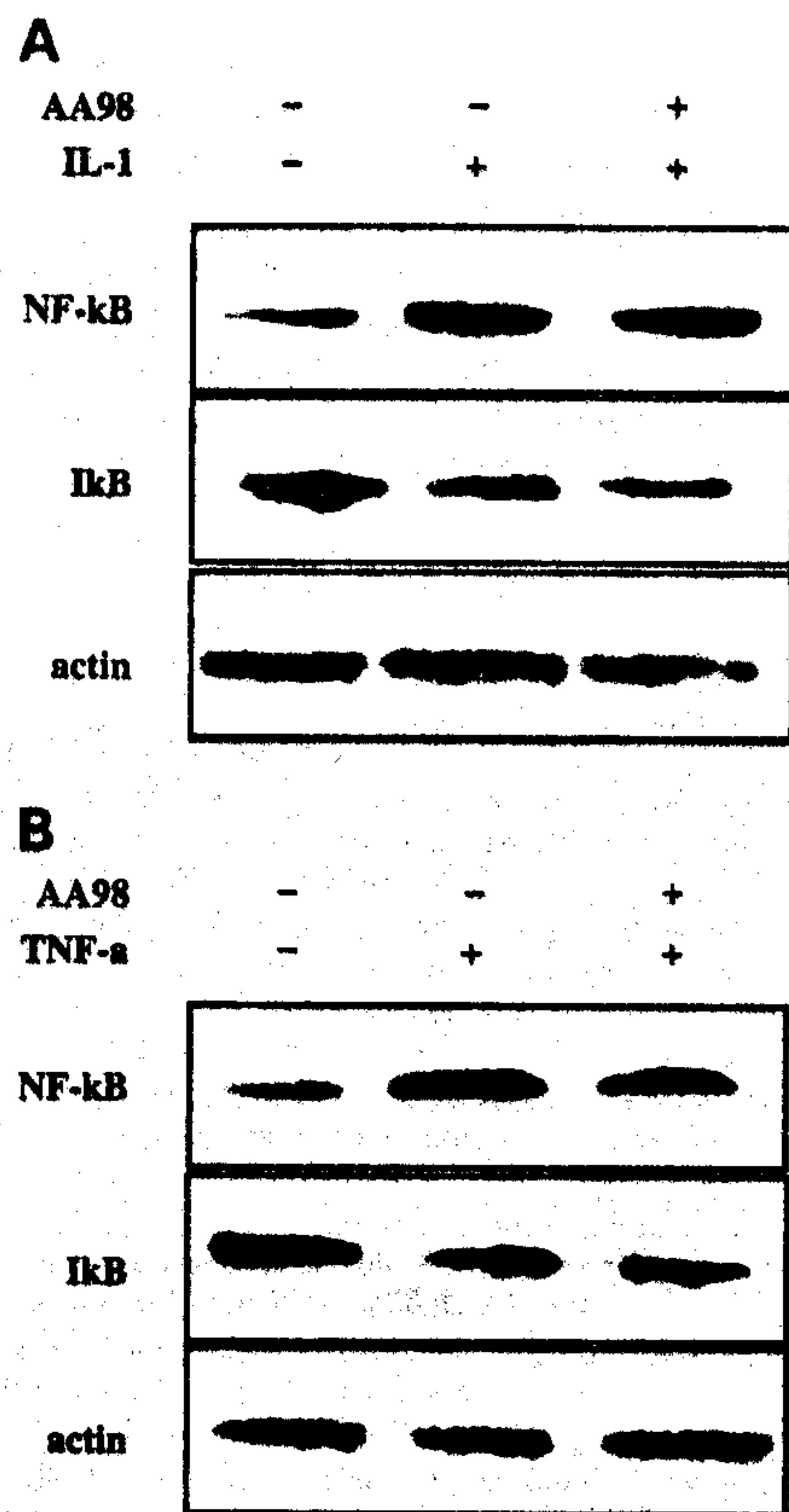
#### mAb AA98 Inhibits MMP-9 Activity and Expression Enhanced by SMMC 7721-CM

It is known that MMP-2 and MMP-9 play a key role in tumor cell invasion, metastasis, and angiogenesis by

promoting extracellular matrix degradation. NF- $\kappa$ B activity has also been associated with the regulation of MMP-9. To determine whether the SMMC 7721-CM-induced NF- $\kappa$ B activation results in the up-regulation of MMP-9 and MMP-2, we did a gelatin zymography assay to analyze the activity of MMP-9 and MMP-2 in the HUVECs treated with SMMC 7721-CM. As shown in Fig. 5A, the activity of MMP-9, but not MMP-2, was enhanced in the HUVECs after stimulation with SMMC



**Figure 2.** mAb AA98 inhibiting NF- $\kappa$ B activity and I $\kappa$ B $\alpha$  degradation in the HUVECs. **A**, confocal imaging shows that mAb AA98, but not control mIgG, inhibited NF- $\kappa$ B (p65 and p50) nuclear translocation in HUVECs which had been stimulated by SMMC 7721-CM. The HUVECs were first stimulated with SMMC 7721-CM for 24 h, and then treated with either 50  $\mu$ g/mL of mAb AA98 or the same amount of mIgG for 1 h. The treated cells were fixed on a coverslip, stained with either anti-p65 or anti-p50 antibody, followed by FITC-conjugated or Cy3-conjugated second antibody, and then observed under a confocal microscope. Western blot shows that mAb AA98 suppressed NF- $\kappa$ B (p65) nuclear translocation and I $\kappa$ B $\alpha$  degradation in a dose-dependent (**B**), and in a time-dependent (**C**) manner. Conversely, the control mIgG did not show any effect on NF- $\kappa$ B (p65) and I $\kappa$ B $\alpha$  activity even at the highest concentration (50  $\mu$ g/mL) and longer time (90 min). The membranes were blotted using anti-p65, anti-I $\kappa$ B $\alpha$ , and anti- $\beta$ -actin antibodies, respectively.  $\beta$ -Actin was used here as an internal control for I $\kappa$ B $\alpha$  in cytosol.



**Figure 3.** mAb AA98 did not affect NF- $\kappa$ B and I $\kappa$ B $\alpha$  activity induced by IL-1 $\beta$  and TNF- $\alpha$ . HUVECs were treated with either IL-1 $\beta$  (10 ng/mL) or TNF- $\alpha$  (10 ng/mL) for 30 min, followed by the addition of mAb AA98 (50  $\mu$ g/mL) for 1 h. Western blotting was done to detect NF- $\kappa$ B (p65) in the nuclei and I $\kappa$ B $\alpha$  in the cytosol. Both factors induced NF- $\kappa$ B activation, although this activation was not inhibited by mAb AA98.

7721-CM, compared with the HUVECs grown in normal DMEM. This increased MMP-9 activity was specifically inhibited by mAb AA98, but was not affected by control mIgG.

To further investigate whether the enhanced MMP-9 activity was due to its up-regulation at the gene transcriptional level, we did a reverse transcription-PCR to analyze the mRNA expression of MMP-9 in the HUVECs with or without the treatment of SMMC 7721-CM. In this experiment, glyceraldehyde-3-phosphate dehydrogenase was used as a control gene. The results indicated that SMMC 7721-CM significantly up-regulated MMP-9 gene expression, and this up-regulated MMP-9 was selectively reduced by mAb AA98 (Fig. 5B). These findings indicate that the expression and activity of MMP-9 were up-regulated by SMMC 7721-CM and were down-regulated by mAb AA98.

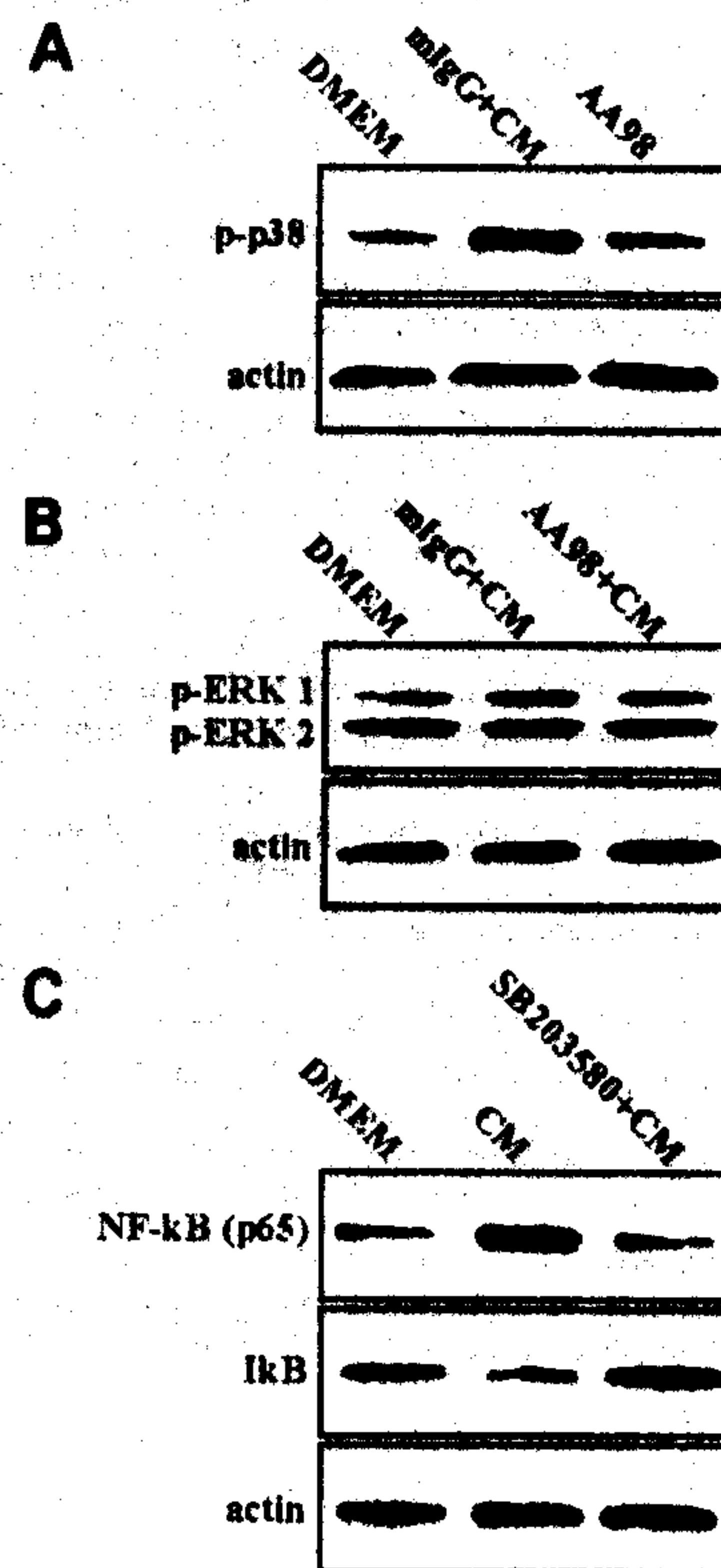
#### mAb AA98 Inhibits ICAM-1 Expression Enhanced by SMMC 7721-CM

NF- $\kappa$ B activity has been associated with the regulation of ICAM-1. To determine whether the SMMC 7721-CM-induced NF- $\kappa$ B activation results in the up-regulation of ICAM-1, we tested the expression of ICAM-1 in the

transcription and protein levels after HUVECs were stimulated with SMMC 7721-CM for 24 hours, and followed by treatment with mAb AA98 for another 24 hours. The results from both Western blot and reverse transcription-PCR showed that SMMC 7721-CM up-regulated the expression of ICAM-1, and that this activation was inhibited by mAb AA98. In contrast, the isotype-matched mIgG did not show any effect on the expression of ICAM-1 in either level (Fig. 6A and B).

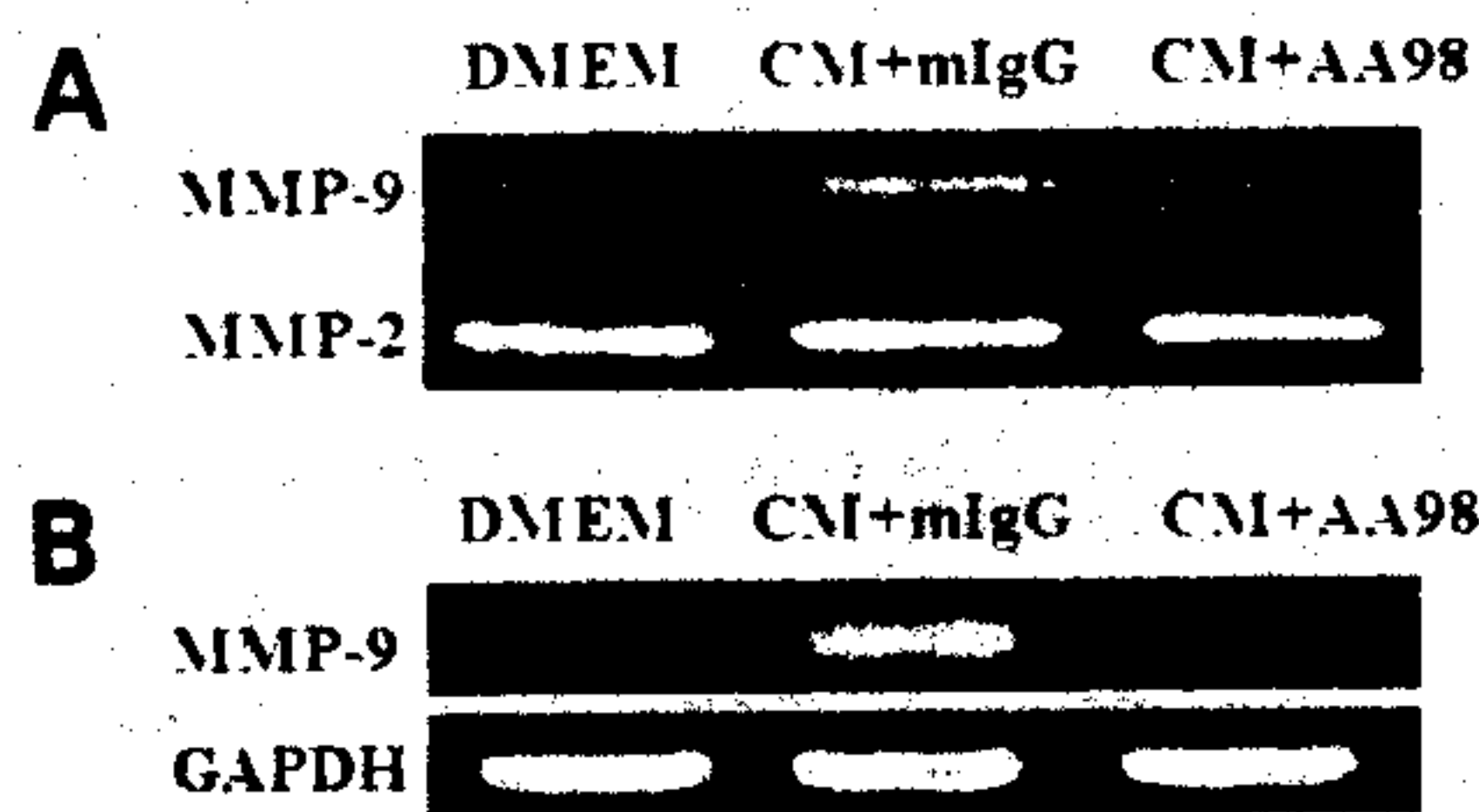
#### Discussion

Our previous study showed that SMMC 7721-CM induced CD146 expression on HUVECs, and promoted HUVEC proliferation and migration. All the SMMC 7721-CM-induced activations of HUVECs could be suppressed by the



**Figure 4.** p38 MAPK phosphorylation induced by SMMC 7721-CM and inhibited by mAb AA98 in HUVECs. HUVECs were first cultured with either SMMC 7721-CM or normal DMEM for 24 h, and then treated with inhibitors (50  $\mu$ g/mL mAb AA98 or 50  $\mu$ g/mL mIgG as control or p38 MAPK inhibitor SB203580) for 1 h. The cell lysates were blotted using anti-p-p38, anti-p-ERK1/2, anti- $\beta$ -actin, and anti-NF- $\kappa$ B (p65) in the nuclei and anti-I $\kappa$ B $\alpha$  in the cytosol.  $\beta$ -Actin was used as an internal control for the cytosol fraction. **A**, SMMC 7721-CM specifically induced the phosphorylation of p38 MAPK, and this p38 MAPK activity could be inhibited by mAb AA98, but not by control mIgG. **B**, ERK MAPK was not affected by SMMC 7721-CM, control mIgG, and mAb AA98. **C**, SMMC 7721-CM promoted NF- $\kappa$ B (p65) translocation into the nuclei and I $\kappa$ B $\alpha$  degradation in the cytosol. This NF- $\kappa$ B activity could be blocked by SB203580, suggesting that NF- $\kappa$ B activation and I $\kappa$ B $\alpha$  degradation either stimulated by SMMC 7721-CM or inhibited by mAb AA98 are mediated by the upstream pathway of p38 MAPK.





**Figure 5.** MMP-9 activated by SMMC 7721-CM and inhibited by mAb AA98 in HUVECs. **A**, zymogram shows that the activity of MMP-9 in HUVECs increased after the cells were cultured with SMMC 7721-CM for 24 h. This increase in MMP-9 activity could be effectively blocked by treatment with 50  $\mu$ g/mL of mAb AA98 for 24 h. However, neither SMMC 7721 nor mAb AA98 showed any effect on MMP-2 activity in the treated cells. **B**, reverse transcription-PCR analysis of MMP-9 expression was up-regulated by SMMC 7721-CM, and inhibited by mAb AA98 in HUVECs. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control.

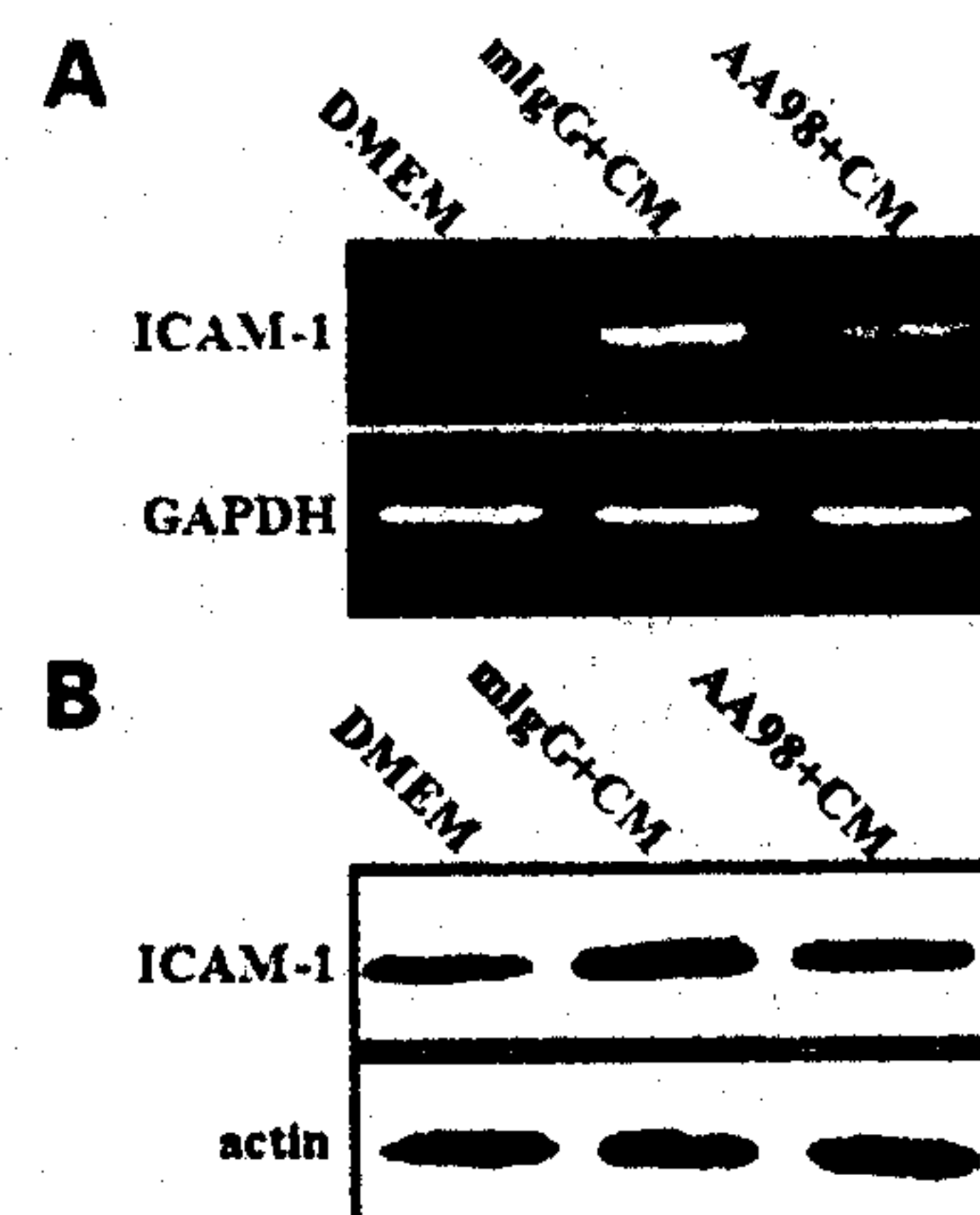
anti-CD146 mAb AA98 (12), such as the cell migration shown in Fig. 7. However, the mechanism behind this has not been elucidated. In this study, we show that the NF- $\kappa$ B activation induced by SMMC 7721-CM plays an important role in the activity of MMP-9, expression of ICAM-1, endothelial cell migration, and angiogenesis.

We confirmed this finding by demonstrating that (a) SMMC 7721-CM induced I $\kappa$ B $\alpha$  degradation in the cytosol and NF- $\kappa$ B translocation into the nuclei, as determined by confocal imaging and biochemical studies; (b) SMMC 7721-CM selectively induced the phosphorylation of p38 MAPK rather than ERK. Moreover, we found that a specific p38 MAPK inhibitor, SB203580, suppressed the I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B translocation which was induced by SMMC 7721-CM. These data support the finding that SMMC 7721-CM-induced NF- $\kappa$ B activation is through the upstream pathway of p38 MAPK; (c) SMMC 7721-CM specifically enhanced both mRNA expression and enzyme activity of MMP-9, but did not affect MMP-2, in the same stimulated HUVECs. These results are consistent with the finding that platelet-activating factor induced angiogenesis through MMP-9 but not through MMP-2 activity (18); (d) the expression of ICAM-1 was enhanced by SMMC 7721-CM and suppressed by mAb AA98 in both mRNA and protein levels; (e) mAb AA98 inhibited endothelial cell migration promoted by SMMC 7721-CM (Fig. 7). All these data support the findings that SMMC 7721-CM promoted endothelial cell migration through its up-regulation of p38 MAPK, NF- $\kappa$ B activation, and overexpression of ICAM-1 and MMP-9 activity.

Another important objective in this study was to clarify the molecular mechanism of the inhibitory functions of mAb AA98 on endothelial cell activation, including migration and angiogenesis (12). Many reports have suggested that the activity of MMP-9 and ICAM-1 are directly associated with cellular migration, invasion, metastasis, and angiogenesis (19, 20). Because MMP-9 and ICAM-1 are both target genes of NF- $\kappa$ B, the inhibition of NF- $\kappa$ B activation could result in the down-regulation of MMP-9 and ICAM-1 expression, which suppressed angiogenesis and tumor

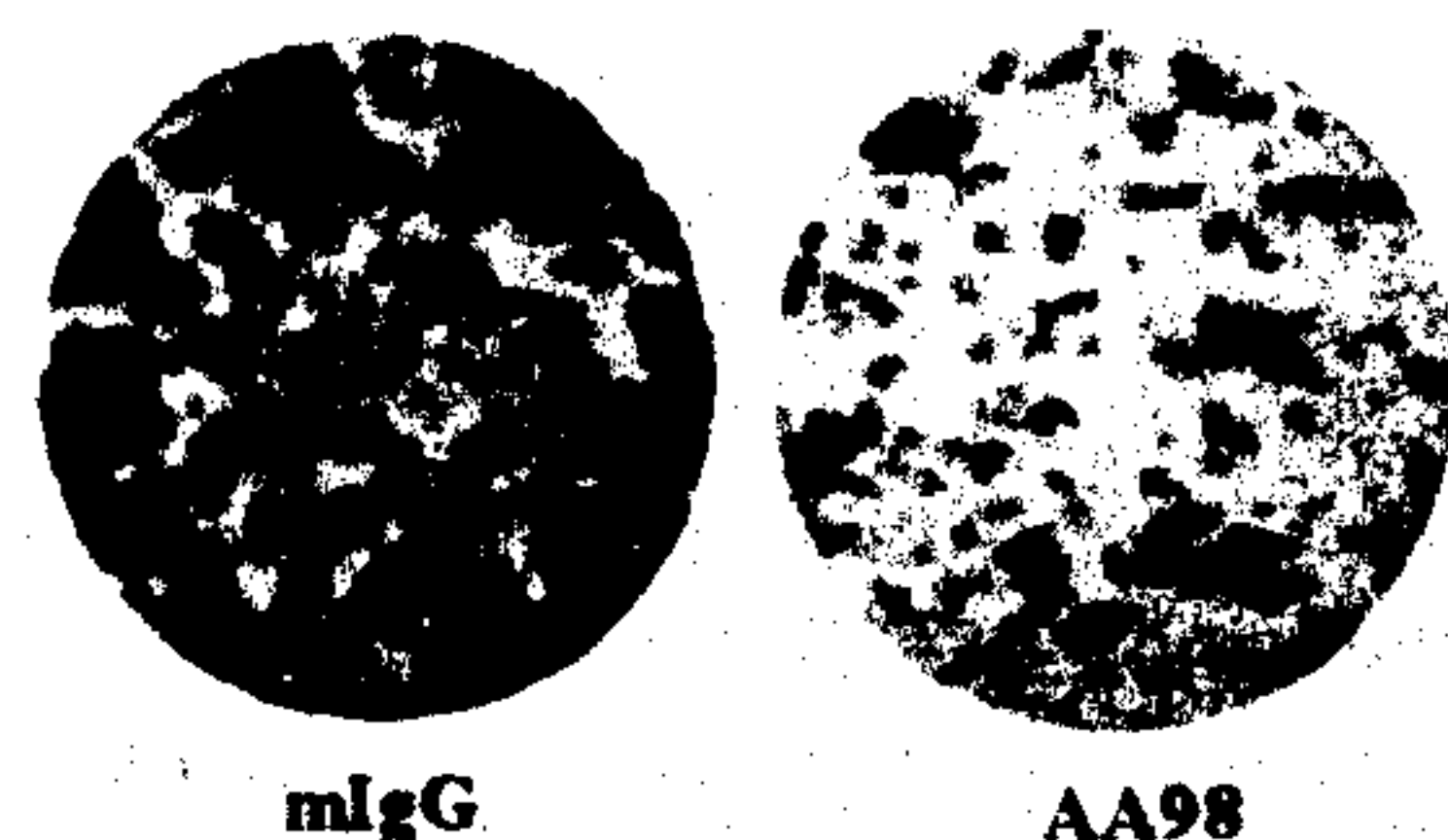
metastasis (18). Our data presents further evidence of NF- $\kappa$ B activation promoting MMP-9 and ICAM-1 expression. In this study, we found that mAb AA98 down-regulated all the activities which had been stimulated by SSMC 7721-CM, including NF- $\kappa$ B activation, p38 MAPK phosphorylation, and overexpression of MMP-9 and ICAM-1. Based on these findings, we speculate that the engagement of mAb AA98 with the cell membrane produces CD146 conformational changes, which inhibit p38 MAPK phosphorylation, leading to the suppression of NF- $\kappa$ B, down-regulation of MMP-9 and ICAM-1, and finally, reducing cell migration and angiogenesis, and tumor metastasis. Although at the moment, we are not able to show the relationship between membrane protein CD146 and the p38 MAPK pathway, several lines of evidence imply that CD146 is involved in signaling. For example, CD146 possesses potential recognition sites for protein kinases in its cytoplasmic tail (9). In addition, endothelial CD146 cross-linked by anti-CD146 antibody induced the recruitment of p59<sup>fyn</sup> kinase to CD146 and increased the phosphorylation levels of p125<sup>FAK</sup> (21). It will be of interest to study the role of CD146 on NF- $\kappa$ B activation and associated signaling pathway.

As far as we know, SMMC 7721-CM contains a number of angiogenic factors and cytokines (12), and NF- $\kappa$ B can be activated by many stimuli, including IL-1 $\beta$  (22) and TNF- $\alpha$  (23, 24). To identify which factor in SMMC 7721-CM is a major contributor in activating NF- $\kappa$ B through the CD146-induced signal pathway, we tested the effects of IL-1 $\beta$  and TNF- $\alpha$ . The results showed that both factors, IL-1 $\beta$  and TNF- $\alpha$ , induced I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B nuclear translocation. However, this activation was not inhibited by mAb AA98. This data indicates that neither IL-1 $\beta$  nor TNF- $\alpha$  is the NF- $\kappa$ B inducer which triggers a signaling pathway related to CD146 and mAb AA98. It is of great interest to identify this factor.



**Figure 6.** Expression of ICAM-1 enhanced by SMMC 7721-CM and inhibited by mAb AA98 in HUVECs. HUVECs were cultured with SMMC 7721-CM for 24 h, followed by treatment with 50  $\mu$ g/mL of mAb AA98 for 24 h. The expressions of ICAM-1 were up-regulated by SMMC 7721-CM and suppressed by mAb AA98 in reverse transcription-PCR (A) and Western blots (B).  $\beta$ -Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls.





**Figure 7.** Boyden chamber assay for the inhibitory function of mAb AA98 on endothelial cell migration. HUVECs were added to the upper chamber of transwells in the presence of AA98 or control mIgG at the same amount (50  $\mu$ g/mL) cultured for 24 h. The migrated cells were stained with Giemsa and counted.

With increasing studies of CD146, we realize that various results come from different laboratories using different anti-CD146 antibodies. For instance, in this report, we found that mAb AA98 inhibited NF- $\kappa$ B activation by blocking the nuclear translocation of p50 and p65. Whereas Solovey et al. reported that P1H12, another anti-CD146 antibody, altered the distribution of NF- $\kappa$ B (p50) but not NF- $\kappa$ B (p65) in HMECs (25). Also, the F(ab')<sub>2</sub> fragment of S-Endo-1 has been reported to recruit fyn in HUVECs via cross-linking CD146 (21). Although we found that mAb AA98 could not recruit fyn, but induces an unknown protein phosphorylated in HUVECs, and the level of phosphorylation seemed to be suppressed in a dose-dependent manner (data not shown). Moreover, ABX-MA1, a fully humanized antibody against CD146, decreased MMP-2 activity and down-regulated the activity of its promoter (10). However, we found that mAb AA98 inhibited the expression and activity of MMP-9 but not MMP-2. A possibility for these contrasting observations is that they may have resulted from different epitopes of the antibodies. The fact that mAb AA98 does not bind its target CD146 in reducing conditions and in paraffin sections, shows its unique character, which is different from other anti-CD146 antibodies.

In summary, this study shows that SMMC 7721-CM-induced NF- $\kappa$ B activation is a critical event in the upstream pathway leading to the induction of MMP-9 and ICAM-1 expression, and furthermore, in endothelial cell migration. In turn, the suppression of NF- $\kappa$ B by mAb AA98 is crucial to its antiangiogenic effect. These data will provide clues for a better understanding of the mechanisms underlying tumor angiogenesis as well as in antiangiogenesis therapy.

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#### References

- Shemirani B, Crowe DL. Head and neck squamous cell carcinoma lines produce biologically active angiogenic factors. *Oral Oncol* 2000;36:61–6.
- Lowrie AG, Salter DM, Ross JA. Latent effects of fibronectin,  $\alpha$ 5 $\beta$ 1 integrin,  $\alpha$ V $\beta$ 5 integrin and the cytoskeleton regulate pancreatic carcinoma cell IL-8 secretion. *Br J Cancer* 2004;91:1327–34.
- Moser TL, Young TN, Rodriguez GC, et al. Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma. *Int J Cancer* 1994;56:552–9.
- Shishodia S, Aggarwal BB. Nuclear factor- $\kappa$ B: a friend or a foe in cancer? *Biochem Pharmacol* 2004;68:1071–80.
- Woessner JF, Jr. MMPs and TIMPs—an historical perspective. *Mol Biotechnol* 2002;22:33–49.
- Antonelli A, Bianchi M, Crinelli R, Gentilini L, Magnani M. Modulation of ICAM-1 expression in ECV304 cells by macrophage-released cytokines. *Blood Cells Mol Dis* 2001;27:978–91.
- Lehmann JM, Riethmuller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. *Proc Natl Acad Sci U S A* 1989;86:9891–5.
- Lehmann JM, Holzmann B, Breitbart EW, Schmiegelow P, Riethmuller G, Johnson JP. Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. *Cancer Res* 1987;47:841–5.
- Sers C, Kirsch K, Rothbacher U, Riethmuller G, Johnson JP. Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. *Proc Natl Acad Sci U S A* 1993;90:8514–8.
- Mills L, Tellez C, Huang S, et al. Fully human antibodies to MCAM/MUC18 inhibit tumor growth and metastasis of human melanoma. *Cancer Res* 2002;62:5106–14.
- Duda DG, Cohen KS, di Tomaso E, et al. Differential CD146 expression on circulating versus tissue endothelial cells in rectal cancer patients: implications for circulating endothelial and progenitor cells as biomarkers for antiangiogenic therapy. *J Clin Oncol* 2006;24:1449–53.
- Yan X, Lin Y, Yang D, et al. A novel anti-CD146 monoclonal antibody, AA98, inhibits angiogenesis and tumor growth. *Blood* 2003;102:184–91.
- Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973;52:2745–56.
- Luca M, Huang S, Gershenwald JE, Singh RK, Reich R, Bar-Eli M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am J Pathol* 1997;151:1105–13.
- Craig R, Larkin A, Mingo AM, et al. p38 MAPK and NF- $\kappa$ B collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system. *J Biol Chem* 2000;275:23814–24.
- Je JH, Lee JY, Jung KJ, et al. NF- $\kappa$ B activation mechanism of 4-hydroxyhexenal via NIK/IKK and p38 MAPK pathway. *FEBS Lett* 2004;566:183–9.
- Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Giombotski CC. MKK6 activates myocardial cell NF- $\kappa$ B and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. *J Biol Chem* 1998;273:8232–9.
- Ko HM, Park YM, Jung B, et al. Involvement of matrix metalloproteinase-9 in platelet-activating factor-induced angiogenesis. *FEBS Lett* 2005;579:2369–75.
- Bergers G, Brekken R, McMahon G, et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2000;2:737–44.
- Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. *Eur J Cancer* 2000;36:1621–30.
- Anfosco F, Bardin N, Frances V, et al. Activation of human endothelial cells via S-endo-1 antigen (CD146) stimulates the tyrosine phosphorylation of focal adhesion kinase p125(FAK). *J Biol Chem* 1998;273:26852–8.
- Chen D, Li X, Zhai Z, Shu HB. A novel zinc finger protein interacts with receptor-interacting protein (RIP) and inhibits tumor necrosis factor (TNF)- and IL1-induced NF- $\kappa$ B activation. *J Biol Chem* 2002;277:15985–91.
- Ariga A, Namekawa J, Matsumoto N, Inoue J, Umezawa K. Inhibition of tumor necrosis factor- $\alpha$ -induced nuclear translocation and activation of NF- $\kappa$ B by dehydroxymethyl epoxyquinomicin. *J Biol Chem* 2002;277:24625–30.
- Hanson JL, Anest V, Reuther-Madrid J, Baldwin AS. Oncoprotein suppression of tumor necrosis factor-induced NF- $\kappa$ B activation is independent of Raf-controlled pathways. *J Biol Chem* 2003;278:34910–7.
- Solovey AN, Gul L, Chang L, et al. Identification and functional assessment of endothelial P1H12. *J Lab Clin Med* 2001;138:322–31.