NF- κ B promotes iNOS and VEGF expression in salivary gland adenoid cystic carcinoma cells and enhances endothelial cell motility *in vitro*

Jiali Zhang*[†] and Bin Peng*

*Key Laboratory for Oral Biomedical Engineering of Ministry of Education and [†]Department of Oral Pathology, School of Stomatology, Wuhan University, Wuhan, China

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Abstract

Tumour cell-derived angiogenic-related factors and endothelial cell mobility are essential for neovascularization and haematogenous metastasis of adenoid cystic carcinoma (ACC) of the human salivary glands. Our previous study demonstrated that vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and nuclear factor kappaB (NF- κ B) staining correlated with ACC microvessel density. However, there still remains a lack of direct evidence to clarify the function of liposaccharide-induced NF- κ B activity in ACC angiogenesis.

Objectives: This study aimed to demonstrate the function of liposaccharide-induced NF- κ B signalling pathway (which would be relevant to angiogenesis) of ACC cell lines.

Materials and methods: A PCMV-I κ B α M vector transfection assay was performed to inhibit NF- κ B activity. Constitutive and liposaccharide-induced NF- κ B activity, along with VEGF and iNOS expression, was detected by electrophoretic mobility shift assays, reverse transcription–polymerase chain reaction and Western blot assay. Tumour cell-derived NO₂⁻ was evaluated by a nitrite determination assay, and endothelial cell mobility was investigated by endothelial cell proliferation, migration and tube formation assays.

Results: We demonstrated that regulation of VEGF and iNOS expression utilized the NF- κ B signalling pathway in ACC cell lines. In the constitutive and liposaccharide-induced condition, NF- κ B activity was blocked by phosphorylation-defective I κ B α .

Accordingly, mRNA and protein expression of VEGF and iNOS were significantly decreased, with a great drop in endogenous NO₂⁻. Furthermore, supernatant from ACC-2/I κ B α M and ACC-M/I κ B α M cells resulted in much lower endothelial cell mobility than that from ACC-2 and ACC-M cells.

Conclusions: Our results suggest that NF- κ B promotes iNOS and VEGF expression in salivary gland ACC cells and enhances endothelial cell motility *in vitro*.

Introduction

Adenoid cystic carcinoma (ACC) of human salivary glands is characterized by high rate of recurrence and early development of haematogenous metastasis. It has a relentless clinical course and usually results in a very poor long-term outcome: only 20% of patients diagnosed with distant metastasis survive 5 years (1). Early studies have demonstrated that because angiogenesis is partly dependent on adequate vascular support formation, it might be a possible mechanism involved in both tumour invasiveness and in aggressive metastatic dissemination (2). Although recent efforts aimed to reduce both tumour growth and spread have largely focused on angiogenesis (3–5), relatively few studies have examined the implication of angiogenic regulation and related factors, as well as their expression in ACC.

Angiogenesis is a process tightly regulated by actions of angiogenic cytokines, with the most notable being vascular endothelial growth factor (VEGF). It is known to exert its mitogenic activity especially on endothelial cells and usually initiates the tumour angiogenic process through recruitment and proliferation of endothelial cells (6). Another angiogenesis modulator, nitric oxide (NO), is a short-lived pleiotropic biomolecule derived from L-arginine by the enzyme inducible NO synthase (iNOS) (7). Compared to a further two isoforms of NO synthase (nNOS, eNOS), there is a growing body of evidence

Correspondence: Bin Peng, Key Laboratory for Oral Biomedical Engineering of Ministry of Education, School of Stomatology, Wuhan University, 237 Luoyu Road, Wuhan 430079, China. Tel.: +862 7871 61981; Fax: +862 7878 73260; E-mail: phs301@vip.163.com

suggesting that output of NO generated by iNOS is higher in malignant tissue and, thus, increase tumour growth rate, vascular density and invasiveness (8). Our previous study concluded that high expression level of VEGF and iNOS significantly correlated with the microvessel density in ACCs and are also considered to be independent prognostic indicators for overall survival (9). Although recent *in vivo* and *in vitro* studies have reported aberrant expression of VEGF and iNOS in ACC cells (10–12), possible regulation pathways of VEGF and iNOS in ACCs is still unclear.

NF- κ B is a nuclear transcription factor that is sequestered in the cytoplasm by I κ B proteins (13). Activation of NF- κ B by cytokines requires phosphorylation and degradation of its cytoplasmic inhibitor I κ B α (14). Recent studies have shown that NF- κ B activity and I κ B α phosphorylation can be detected in cells of many neoplasms, including ACCs (12,15–17). Activated NF- κ B is reported to play a key role in malignant neoplasm angiogenesis. It has also been found that free NF- κ B dimers translocate to the nucleus and up-regulate a number of genes necessary for tumour angiogenesis, such as VEGF, iNOS, cytochrome *c* oxidase subunit II, interleukin-6, interleukin-8, and a urokinase-type plasminogen activator, to name but a few, which have κ B binding sites in their promoter regions (18). Hence, blocking NF- κ B activity is assumed to interfere with the tumour angiogenesis process by suppressing angiogenic-related factors.

Our previous study demonstrated that the NF- κ B nuclear localization was associated with VEGF and iNOS aberrant expression in both clinical ACC tissue samples and ACC cell lines (9,12). In addition, NF- κ B activity, VEGF, and iNOS expression also correlated with both microvessel density in ACC tissues and ACC cell lines *in vitro* angiogenesis (9). Nevertheless, the role of the liposaccharide-induced NF- κ B signalling pathway in VEGF and iNOS expression in human ACC angiogenesis is still unclear. Therefore, the present study was designed to demonstrate (i) whether the NF- κ B signalling pathway is directly involved in regulation of constitutive and liposaccharide-induced VEGF and iNOS expression in ACCs, and (ii) whether blocking the NF- κ B signalling pathway in ACC cells interferes with tumour angiogenesis through decrease in motility of endothelial cells.

Materials and methods

Stable transfection of ACC cells with I κ B α M and control vector

The PCMV-I κ B α M vector was provided by Professor Chiao (Anderson Cancer Center, Houston, TX, USA); it has mutations (S32, 36A) of the NH2 terminus and a COOH-

terminal PEST sequence, which specifically inhibits phosphorylation of I κ B α . ACC-M and ACC-2 cells (1×10^6) were transfected using 15 μ l of lipofectamine reagent (Invitrogen, Grand Island, NY, USA), and 4 μ g of PCMV-I κ B α M or a control PCMV vector, according to the manufacturer's instructions. Cells were selected using a standard medium containing 800 μ g/ml and 600 μ g/ml G418, respectively. Fourteen days later, neo-resistant colonies were isolated by trypsinization and were established as subcultures. The cells that resisted G418 and expressed stable exogenous I κ B α M were used for subsequent analyses.

Cell culture and liposaccharide stimulation

High and low metastasis cell lines of human ACC (ACC-2 and ACC-M) were obtained from the China Center for Type Culture Collection (20). The low metastasis cell line (ACC-2) was established from ACC (19), and the high metastasis cell line (ACC-M) was a highly metastatic clone to the lung selected from ACC-2. Metastatic rates were 96% vs. 18% for the ACC-M and ACC-2 cell line (20). Umbilical vein endothelial cell (UEVC) line was provided by the Key Laboratory for Oral Biomedical Engineering of Ministry of Education at Wuhan University, China. Cells were all cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in 5% CO₂ and 95% air atmosphere.

ACC-2 and ACC-M cells were plated in 6-well plates. When cells reached a density of 1×10^6 cells/well, they were activated by incubation with a medium containing *Escherichia coli* liposaccharide (Sigma-Aldrich, St. Louis, MO, USA) for 0, 6, 12, 24 or 48 h, concentration 100 ng/ml.

To obtain the conditioned medium (CM), subconfluent ACC-M, ACC-2, ACC-M/I κ B α M, and ACC-2/I κ B α M cells were serum starved for 12 h in 6-well plates (Greiner, Labortechnik, Germany). Medium was replaced with 1.0-ml serum-free DMEM. Then cells were incubated for 12 h, after which CM were obtained, stored at -20°C , and used for the following experiments.

Electrophoretic mobility shift assay

Nuclear extraction kit (Chemicon International Inc., Temecula, CA, USA) was used to isolate nuclear proteins. Electrophoretic mobility shift assays (EMSA) were performed as follows: 10 μ g of nuclear extract in a 10- μ l reaction volume was incubated on ice for 40 min. Double-stranded oligodeoxyribonucleotide DNA probes (κ B: 5'-AGTT-GAGGGGACTTTCCAGGC-3', and Oct-1: 5'-TGTC-GAATGCAAATCACTAGAA-3') were end-labelled with ³²P- γ and applied to a 4% nondenatured polyacrylamide

gel. Equal loading of nuclear extracts was confirmed by determining Oct-1 DNA binding activity. For competition assays, a 100-fold molar excess of unlabelled oligonucleotides was added to the binding reaction. For supershift assays, 2 µl of polyclonal antibodies against p65 and p50 were added to the reaction mixture on ice, 40 min before probe was added. After electrophoresis, gel was dried for 1 h at 80 °C and exposed to Kodak X-ray film (Eastman Kodak Co., Rochester, NY, USA) at -80 °C.

Semiquantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from 1×10^6 of ACC-M and ACC-2 cells using TRIzol (Invitrogen). Aliquots (1 µg) of RNA were reverse transcribed to cDNA and aliquots (4 µl) of cDNA were used as a template for polymerase chain reaction (PCR) using a PE9700 RT-PCR system (Applied Biosystems, Singapore) according to the manufacturer's instructions. Primers and thermocycling reaction conditions are shown in Table 1. PCR products were analysed by agarose gel electrophoresis and relative RNA content was calculated using the GeneGenius gel imaging system (Syngene, Cambridge, UK). All experiments were performed three times, with each reading taken in triplicate. Average and standard deviations were then calculated.

Western blot analysis

A nuclear extraction kit (Chemicon International) was used to isolate cytoplasmic proteins. Fifty micrograms of protein of different groups were boiled for 5 min in sample buffer and were then separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10–15%) and transferred on to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Nonspecific reactivity was blocked using 5% bovine serum albumin in a TBST buffer (100 ml 10× TBS, 10 ml 10% Tween20 final 0.1% v/v, dissolve with 890 ml double distilled-H₂O) and then subsequently incubated with primary antibodies anti-

VEGF, anti-iNOS or anti-IκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Membranes were also probed with anti-β-actin (Santa Cruz Biotechnology) to assure equal amounts of protein. Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA). Western blot experiments were repeated at least twice to confirm the results.

Nitrite determination

NO release was measured as nitrite accumulation in the medium using a Griess reagent system kit (Promega, Madison, WI, USA). Briefly, 100 µl of supernatant was incubated with an equal volume of Griess reagent (a combination of equal amounts of 0.1% naphthyl ethylenediamine dihydrochloride in water and 2% sulphanilamide in 5% H₃PO₄), at room temperature for 10 min. Then, absorbance at 540 nm was measured using Ultra Microplate Reader Elx808 (Bio-Tek Instruments, Winooski, VT, USA). Nitrite concentrations were calculated using a NaNO₂ standard curve.

Cell proliferation assay

Cell proliferation assays were performed by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). UVECs were seeded into 96-well plates at 1×10^3 per well in a 100-µl cell culture medium and maintained at 37 °C in a humidified incubator containing 5% CO₂ for 24 h. Cells were starved for 24 h by replacing the media with a serum-free medium. That medium was then replaced by 80 µl CM from ACC-M, ACC-2, ACC-M/IκBaM, and ACC-2/IκBaM cells, which was added to 20 µl of 10% FBS DMEM to reach 2% FBS-CM final concentration. One hundred microlitres of 2% FBS medium was used as control. After 96 h culture, 10 µl of CCK-8 solution was added to the triplicate wells and incubated for 3 h. Then, absorbance at 450 nm was measured to calculate number of vital cells in each well. Cell proliferation is represented as the mean percentage ± standard deviation of absorbance.

Table 1. Primers and thermocycling reaction conditions

	Primers	Product size (bp)	Annealing temperature	Cycles
VEGF	5'-GGC TCT AGA TCG GGC CTC CGA AAC CAT-3' 5'-GGC TCT AGA GCG CAG AGT CTC CTC TTC-3'	682	63.5 °C	27
iNOS	5'-TGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3' 5'-GGT GCT ACT TGT TAG GAG GTC AAG TAA AGG GC-3'	253	59 °C	30
β-actin	5'-TGT GCC CAT CTA CGA GGG GTA TGC-3' 5'-GGT ACA TGG TGG TGC CGC CAG ACA-3'	434	57 °C	25

Wound healing assay

Endothelial cell motility assays were carried out as previously described (21). Briefly, 6-well plates were coated with type I collagen and incubated overnight at 37 °C. UVECs were seeded into the coated wells at a density of 2×10^6 cells/well and incubated for 24 h. Then the monolayer of cells was scraped, to provide a clear area, with a narrow tip and was washed with a serum-free medium. One millilitre of CM from ACC-M, ACC-2, ACC-M/I κ BaM and ACC-2/I κ BaM cells was added to the wells and incubated for 24 h, (1.0 ml serum-free DMEM serving as the control). Cells were then fixed and stained using acridine orange and photographed using a fluorescence microscope (Leica Microsystems, Heidelberg, Germany). Cell movement in the gap area was evaluated by the following criteria: 0, no cell movement in the wound area; 1+, <25% of the wound area filled by cell movement; 2+, between 25% and 50% of the wound area filled by cell movement; 3+, between 50% and 75% of the wound area filled by cell movement; and 4+, >75% of the wound area filled by cell movement.

Tube formation assay

500- μ l type I collagen gel solution (0.3%) containing 5 mg/ml human fibronectin (Collaborative Research Inc., Lexington, MA, USA), 100 μ l 10 $\times$ DMEM and 400 μ l of NaOH-HEPES buffer was mixed in ice-cold conditions, was pipetted into 6-well plates and kept for 30 min at 37 °C or until gelatinization. Next, UVECs were seeded into a layer of the gel at a density of 5×10^4 cells/well with 10% FBS medium. After 24 h, the medium was replaced by 0.8 ml of CM from ACC-M, ACC-2, ACC-M/I κ BaM and ACC-2/I κ BaM cells, which was added to 0.2 ml 10% FBS DMEM to reach 2% FBS–CM final concentration. One millilitre of 2% FBS medium was used as control (22). After 3 days, tube structures organized and gradually elongated and formed networks of UVEC cells. Then structures were fixed and stained using acridine orange and were photographed using a fluorescence microscope (Leica Microsystems). Mean number of tubes

was quantified by counting branching points per high power field.

Statistical evaluation

All experiments were repeated in triplicate, and all data are presented as mean $\pm$ standard error. Analyses of variance, Student–Newman–Keuls test, and homogeneity-of-variance test were used for statistical analysis. *P*-values less than 0.05 were considered statistically significant.

Results

Liposaccharide effect on NF- κ B activity per time-dependent manner

Constitutive NF- κ B activity was detected in high metastasis cell line ACC-M and in low metastasis cell line ACC-2, as shown in Fig. 1a (lanes 1, 2). Supershift and competition assays showed presence of P65 and P50 in NF- κ B binding activity inside ACC-M cells (Fig. 1b). In response to 100 ng/ml of liposaccharide stimulation, NF- κ B activity was greatly enhanced and peaked at 12 h in both cell lines. NF- κ B binding activity then gradually decreased after being exposed to 100 ng/ml of liposaccharide for 24 and 48 h (Fig. 1c).

Liposaccharide-induced NF- κ B activity correlated with the expressions of VEGF and iNOS

Data presented here show that increased tendency of VEGF and iNOS expression correlated with NF- κ B activity when stimulated with 100 ng/ml of liposaccharide in ACC-M and ACC-2 cell lines (Fig. 2a). Consistent with NF- κ B activity, mRNA levels of VEGF and iNOS increased and peaked at 12 h in both cell lines. After being stimulated with liposaccharide for 24 h, mRNA levels began to decrease slightly; forty-eight hours later, mRNA levels decreased significantly (Table 2). Moreover, VEGF and iNOS protein levels gradually increased in both high and low metastasis cell lines when treated with liposaccharide for 12 h. After

Table 2. mRNA expression level of VEGF and iNOS of ACC cell lines in response to 100 ng/ml liposaccharide at different time points

		Mean $\pm$ SE				
		0 h	6 h	12 h	24 h	48 h
VEGF	ACC-M	0.56 $\pm$ 0.04	1.28 $\pm$ 0.08	2.68 $\pm$ 0.09*	2.16 $\pm$ 0.13*	1.38 $\pm$ 0.29
	ACC-2	0.32 $\pm$ 0.01	1.11 $\pm$ 0.10*	2.25 $\pm$ 0.16*	1.46 $\pm$ 0.22*	1.00 $\pm$ 0.11*
iNOS	ACC-M	0.54 $\pm$ 0.02	0.92 $\pm$ 0.24	2.10 $\pm$ 0.21*	1.99 $\pm$ 0.14*	1.23 $\pm$ 0.26*
	ACC-2	0.26 $\pm$ 0.02	0.59 $\pm$ 0.03	1.79 $\pm$ 0.10*	1.41 $\pm$ 0.09*	0.91 $\pm$ 0.21*

* *P* < 0.05

Figure 1. EMSA showed activity of the NF- κ B in ACC cell lines. (a) Lanes 1 and 2 show constitutive NF- κ B activity in ACC-M and ACC-2 cell lines. Lanes 3 and 4 show NF- κ B activity was completely abolished in ACC-M/I κ B α M and ACC-2/I κ B α M cell lines. (b) Supershift and competition EMSA assays of the NF- κ B activity. Supershift assay (lanes 1–3) show presence of P65 and P50 in the NF- κ B binding activity inside the ACC-M cells. Competition assay (lane 4) show excess of unlabelled oligonucleotides competitively inhibited the binding reaction. (c) In response to 100 ng/ml of liposaccharide stimulation, NF- κ B activity was greatly enhanced and peaked at 12 h in both cell lines. Binding activity then gradually decreased at 24- and 48-h postdose period. The Oct-1 motif was used as control for quality and quantity of cell extract.

Table 3. NO₂ concentration of ACC cell lines in response to 100 ng/ml liposaccharide at different time points

	NO ₂ concentration (μg/ml) (mean ± SE)				
	0 h	6 h	12 h	24 h	48 h
ACC-M	6.15 ± 0.55	10.54 ± 1.23*	20.22 ± 1.76*	13.02 ± 1.12*	8.67 ± 0.20
ACC-2	5.15 ± 0.52	9.84 ± 0.48	16.61 ± 1.56*	11.98 ± 2.55*	8.38 ± 0.68

* $P < 0.05$

being stimulated with liposaccharide for 24 h, VEGF and iNOS protein levels began to decrease (Fig. 2b). In addition, endogenous NO₂⁻ production in the supernatant of ACC-M and ACC-2 cells increased rapidly after incubation with liposaccharide and was close to maximum at 12 h (Fig. 2c; Table 3).

Inhibition of constitutive and liposaccharide-induced NF- κ B activity by I κ B α M

To evaluate the effect of I κ B α M on NF- κ B activity in the ACC cell lines, ACC-M and ACC-2 cells were stably transfected with the mutant I κ B α expression vector

Figure 2. Liposaccharide-induced mRNA and protein expression levels of VEGF and iNOS in ACC cell lines. (a) Liposaccharide-induced mRNA expression levels of VEGF and iNOS were detected by RT-PCR. Bars represent the mean value of VEGF and iNOS mRNA levels with 100 ng/ml liposaccharide stimulation at various time points. The difference of mRNA levels between 0 h and other time points were compared by one-way ANOVA and Student–Newman–Keuls test ($*P < 0.05$). (b) Western blot analysis showed that the protein levels of VEGF and iNOS in ACC-M and ACC-2 cells with 100 ng/ml liposaccharide stimulation at various time points. Protein levels of VEGF and iNOS in ACC-2 and ACC-M cell lines were highest at 12 h after the cell lines were treated with liposaccharide. β -actin was used as control for quality and quantity of cell protein. (c) Bars represent mean value of NO₂⁻ production in the medium of ACC-M and ACC-2 with 100 ng/ml liposaccharide stimulation at various time points. Difference of NO₂⁻ concentration between 0 h and other time points were compared by one-way ANOVA and Student–Newman–Keuls test ($*P < 0.05$).

(IkB α M). The transfected cell lines, ACC-M/IkB α M and ACC-2/IkB α M, expressed both endogenous and mutant IkB α , as shown in Fig. 3a. Endogenous IkB α was detected in parental, control PCMV-transfected, and IkB α M-transfected

cells. However, the exogenous mutant IkB α was detected only in ACC-M/IkB α M and ACC-2/IkB α M cells. Compared to parental cells, constitutive NF- κ B binding activity was almost abolished in ACC-M/IkB α M and ACC-2/IkB α M

Figure 3. (a) Western blot assay showing endogenous IκBα was detected in the parental (lanes 2, 4), control PCMV-transfected (Neo, lanes 5, 6), and IκBαM-transfected cells (lanes 1, 3). Exogenous Tag2A-IκBαM was detected only in the ACC-M/IκBαM and ACC-2/IκBαM cells (lanes 1, 3). (b) Protein expression levels of VEGF and iNOS induced by liposaccharide for 12 h in ACC-2, ACC-M, ACC-M/IκBαM and ACC-2/IκBαM cells. Liposaccharide-induced VEGF and iNOS protein expressions were effectively inhibited by an overexpression of IκBαM. (c) Liposaccharide-induced NF-κB DNA binding activities (lanes 1, 2) were remarkably inhibited by an ectopic expression of IκBαM in ACC-M/IκBαM and ACC-2/IκBαM cells (lanes 3, 4). (d) The constitutive mRNA expression of VEGF and iNOS was observed in ACC-M and ACC-2 cell lines, while it was hard to detect in ACC-M/IκBαM and ACC-2/IκBαM cell lines. (e) VEGF and iNOS mRNA expression in ACC-M/IκBαM and ACC-2/IκBαM cells was significantly lower than that in ACC-2 and ACC-M cells after treated with liposaccharide for 12 h. (f, g) Bars represent the mean value of VEGF and iNOS mRNA levels with 100 ng/ml liposaccharide stimulation for 12 h. The difference of VEGF and iNOS mRNA levels between untransfected and transfected cell lines were compared by one-way ANOVA (* $P < 0.05$).

Table 4. mRNA expression level of VEGF and iNOS of ACC cell lines in response to 100 ng/ml liposaccharide for 12 h

		Mean $\pm$ SE				
		0 h	6 h	12 h	24 h	48 h
VEGF	ACC-M	0.56 $\pm$ 0.04	1.28 $\pm$ 0.08	2.68 $\pm$ 0.09*	2.16 $\pm$ 0.13*	1.38 $\pm$ 0.29
	ACC-2	0.32 $\pm$ 0.01	1.11 $\pm$ 0.10*	2.25 $\pm$ 0.16*	1.46 $\pm$ 0.22*	1.00 $\pm$ 0.11*
iNOS	ACC-M	0.54 $\pm$ 0.02	0.92 $\pm$ 0.24	2.10 $\pm$ 0.21*	1.99 $\pm$ 0.14*	1.23 $\pm$ 0.26*
	ACC-2	0.26 $\pm$ 0.02	0.59 $\pm$ 0.03	1.79 $\pm$ 0.10*	1.41 $\pm$ 0.09*	0.91 $\pm$ 0.21*

* $P < 0.05$

cells (Fig. 1a, lanes 3, 4). Moreover, NF- κ B DNA binding activities induced by 100 ng/ml of liposaccharide at 12 h were remarkably inhibited by ectopic expression of I κ B α M in ACC-M/I κ B α M and ACC-2/I κ B α M cells (Fig. 3c).

Decreased constitutive and liposaccharide-induced VEGF and iNOS expression by blocking NF- κ B activity

Constitutively expressed VEGF and iNOS mRNAs were difficult to detect in the ACC-M/I κ B α M and ACC-2/I κ B α M cell lines, while high expression was observed in the untransfected cell lines (Fig. 3d). In response to liposaccharide stimulation, VEGF and iNOS mRNA expression was still very weak in I κ B α M vector-transfected ACC cell lines. Compared to untransfected cells, there was significant decrease in VEGF mRNA expression in ACC-M/I κ B α M and ACC-2/I κ B α M cells after treatment with liposaccharide for 12 h (Fig. 3e,f; Table 4). Consistently, VEGF protein levels were very weak in liposaccharide-induced ACC-M/I κ B α M and ACC-2/I κ B α M cells (Fig. 3b). Remarkable decrease of iNOS mRNA expression was also detected in ACC-M/I κ B α M and ACC-2/I κ B α M cells after liposaccharide treatment for 12 h (Fig. 3e,g); iNOS protein was expressed weakly in liposaccharide-induced ACC-M/I κ B α M and ACC-2/I κ B α M cells (Fig. 3b). Furthermore, endogenous NO level in supernatant of ACC-M/I κ B α M and ACC-2/I κ B α M cells was almost undetectable (data not shown).

Supernatant from transfected ACC cell lines decreased endothelial cell proliferation

As shown in Fig. 4A, CM from ACC-M and ACC-2 cell lines greatly stimulated endothelial cell proliferation. The most notable proliferation activity of UVECs was a result of exposure to ACC-M supernatant. Furthermore, viability of endothelial cells plummeted when stimulated with the supernatant from transfected cell lines ACC-2/I κ B α M and ACC-M/I κ B α M. However, there was no statistical difference in UVEC proliferation between those stimulated with supernatants from either ACC-M/I κ B α M and ACC-M/I κ B α M. Endothelial cells cultured with control medium

showed much lower proliferative activity than those cultured in conditioned media from transfected or untransfected cell lines.

Supernatant from transfected ACC cell lines decreased endothelial cell mobility

After 24 h in culture, scrape-wounded UVEC monolayers incubated with 1.0 ml supernatant from ACC-M and ACC-2 cells revealed that endothelial cells migrated into the denuded area more densely than those incubated with the supernatant from ACC-2/I κ B α M and ACC-M/I κ B α M cells (Fig. 4Bb–e). On the other hand, when cultured with 1.0 ml of serum-free DMEM, the cells sparsely migrated into the denuded area and the ‘wound’ remained open (Fig. 4Ba). There was significant increase in migration of UVECs when cells had been exposed to ACC-M and ACC-2 supernatants, compared to cells exposed to control medium. Supernatants from ACC-M/I κ B α M and ACC-2/I κ B α M inhibited migration of UVECs by 66.7% and 62.1%, respectively (Fig. 4C).

Supernatant from transfected ACC cell lines decreased endothelial cell tube formation

In the presence of 2% FBS-CM from ACC-M, UVECs formed organized elongated tube-like structures resembling an extensive network of capillaries (Fig. 4Bg). In the presence of 2% FBS-CM from ACC-2, tube-like UVEC formations and networks could also be observed, although the tube-like structures stimulated by ACC-2 supernatant appeared to be fewer than those stimulated by ACC-M supernatant (Fig. 4Bh). However, when cultured with supernatants from ACC-2/I κ B α M and ACC-M/I κ B α M cells, tube formation was diminished and only a few well-organized structures were observed (Fig. 4Bi,j); endothelial cells cultured with control medium were poorly organized (Fig. 4Bf). Furthermore, the ACC-M/I κ B α M and ACC-2/I κ B α M supernatants showed, respectively, a 63.3% and 68.4% inhibition of tube formation compared to in ACC-M and ACC-2 supernatants (Fig. 4D).

Figure 4. Endothelial cell proliferation, migration and tube formation assays. (A) Bars represent mean absorbance of 540 nm when endothelial cells were treated with different conditioned media for 96 h. Supernatant from transfected ACC cell lines decreased endothelial cell proliferation. (B) UVECs were cultured with control conditioned medium (a), ACC-M (b), ACC-2 (c), ACC-M/IκBα (d), and ACC-2/IκBα (e) for mobility assay. Edges of denuded areas are marked by dashed lines. UVECs were cultured with the conditioned medium from the control (f), ACC-M (g), ACC-2 (h), ACC-M/IκBα (i), and ACC-2/IκBα (j) for tube formation assay. One of the tube-like structures is marked by an asterisk (*) in (g) and (h). (C) Bars represent the mean scores of endothelial cell migration calculated by evaluating the gap filled area. It was shown that the supernatant from the transfected ACC cell lines decreased endothelial cell mobility. (D) Bars represent the mean number of branching points per high power field. It was shown that the supernatant from transfected ACC cell lines decreased endothelial cell tube formation.

Discussion

This study provides new insight into a potential angiogenic-related signalling pathway in ACCs. The data presented show that liposaccharide affected NF- κ B activity in a time-dependent manner in both high and low metastasis ACC cell lines (ACC-M and ACC-2). It appears that both VEGF and iNOS mRNA regulation and protein expression utilized the NF- κ B signalling pathway. Blocking NF- κ B activity in ACC cells interfered with 'angiogenesis' by decreasing motility of endothelial cells. These results provide evidence that NF- κ B might be a promising focal point in the ACC angiogenic process.

It is well recognized that in general, chronic inflammation can incite a malignant change. NF- κ B, a nuclear transcription factor, has been proven to play an important role in inflammation-induced tumour development. A wide range of stimuli is involved in regulation of NF- κ B activation, which includes cytokines and viral and bacterial products; much previous literature has reported that liposaccharide activates NF- κ B through indirect ways. Among these, liposaccharide-induced tumour necrosis factor- α (TNF- α), secreted by tumour-associated macrophages, is the most notable. Liposaccharide-induced TNF- α is an important activator of tumour cell NF- κ B in a majority of cancers studied *in vitro* and *in vivo* (7,23). TNF- α also plays a crucial role of in tumour progression that has been fully elucidated by other researchers (7,24). However, some evidence suggests that liposaccharide can activate NF- κ B directly through a toll-like receptor 4 (TLR4) pathway in both normal and tumour cells. Frantz *et al.* have proven that CD4/TLR4 results in liposaccharide-induced NF- κ B activity in cardiac myocytes (25). More recently, the study of Kundu *et al.* demonstrates that liposaccharide directly activates NF- κ B *via* the TLR4 pathway, which induces carcinogenesis of prostate epithelial cells. There, NF- κ B activation was both concentration- and time-dependent when stimulated with liposaccharide (26). These findings coincide with our results. In the present study, compared to constitutive activation, NF- κ B activity increased in a time-dependent manner in both cell lines when treated with 100 ng/ml liposaccharide. Since TLR4 has been reported to be detected in both normal and malignant salivary gland cells (27,28), we assume that the TLR4 might be involved in liposaccharide-induced NF- κ B activity in ACC cell lines.

VEGF is one of the most potent angiogenic factors that can be regulated by activated NF- κ B, because its genes have a κ B binding site in its promoter region (29,30). Our study demonstrated concordant increase in NF- κ B activity in response to liposaccharide stimulation with the elevated VEGF mRNA and protein. It is suggested that liposaccharide-induced NF- κ B activity might play an important role in

VEGF regulation, which might also be involved in ACC angiogenesis. To further investigate the role of NF- κ B activity on constitutive and liposaccharide-mediated VEGF gene expression, we inhibited NF- κ B activity by stable expression of a phosphorylation-defective I κ B α mutant (S32, 36A). Ectopic expression of I κ B α M effectively abolished constitutive and liposaccharide-induced NF- κ B activity, concomitant with significant decrease in VEGF mRNA and protein expression. Our results indicate that constitutive or liposaccharide-induced expression of VEGF is NF- κ B dependent and blocking NF- κ B signalling effectively reduced VEGF expression in the ACC cells. This conclusion is supported by findings of other authors, such as Okamoto *et al.* (31), who demonstrated that inhibitors of transcription factor NF- κ B completely prevented advancement of glycation end products (16) and induced up-regulation of VEGF mRNA, which resulted in DNA synthesis in endothelial cells. In pancreatic cancer, inhibiting NF- κ B activation by expressing I κ B α M significantly reduced *in vivo* expression of VEGF, and, hence, decreased neoplastic angiogenesis (32). In accordance with the previous study and our recent research, it has been demonstrated that regulation of VEGF through the NF- κ B signalling pathway is mediated at the transcription level, which then affects protein expression. NF- κ B signal blockage might be involved in angiogenesis inhibition through down-regulation of VEGF expression in ACC cell lines.

Recently, a series of studies has indicated that NO (produced by iNOS) induces angiogenesis, which in turn aids tumour growth, invasion and metastasis (8,33). In this study, it was found that aberrant iNOS expression and endogenous NO₂⁻ production were higher in the high metastasis cell line. Moreover, variation of iNOS expression was consistent with changes in VEGF level in ACC cells when stimulated with liposaccharide. The results suggest that iNOS expression and endogenous NO production might be involved in ACC angiogenesis. It is also believed that endogenous NO produced by iNOS could activate VEGF function and promote proliferation of endothelial cells (34–36). The iNOS and NF- κ B signalling pathways are also reported to be intimately linked, as *iNOS* is an NF- κ B inducible gene (37). Furthermore, Kim *et al.* found that epicatechin significantly reduced IL-1 β -induced iNOS protein and mRNA expressions by down-regulating NF- κ B activation (38). Our study found that NF- κ B activation correlated with iNOS mRNA and protein expression along with endogenous NO production with liposaccharide stimulation. Block of NF- κ B activity significantly decreased iNOS expression and NO production in both ACC cell lines. These results imply that NF- κ B activation is responsible for NO production and the *iNOS* gene, along with protein expression, in ACCs when stimulated by liposaccharide.

The angiogenic process includes endothelial cell activation, proliferation, migration, tube formation and capillary sprouting (2). To investigate function of the NF- κ B signalling pathway in the ACC angiogenesis process, we performed *in vitro* endothelial cell proliferation, mobility and tube formation assays. Our results showed that supernatant from ACC-2/I κ BaM and ACC-M/I κ BaM cells resulted in much lower endothelial cell proliferation, mobility and tube formation than that from ACC-2 and ACC-M cells. The results demonstrated that NF- κ B inhibition in ACC cells resulted in lower endothelial cell mobility. NF- κ B activation block also significantly decreased VEGF and iNOS expression levels in transfected ACC cells. This indicated that decrease in VEGF and NO level resulting from NF- κ B inhibition might be the possible factors for decreased endothelial cell mobility. Tumour cell-derived angiogenic factor, VEGF, is essential for tumour neovascularization. NO has also been shown to mediate angiogenesis by direct and indirect mechanisms as follows. First, NO exposure increases DNA synthesis, cell proliferation and migration of endothelial cells through the sGC-cGMP pathway (39). Second, NO has been shown to mediate the function of many angiogenic factors (40), such as VEGF. Besides this, several studies have shown that combining anti-VEGF treatments with other treatment results in a greater antitumour angiogenesis effect (19). When we combine the findings of others with our present study, we conclude that blocking the NF- κ B signalling pathway in ACC cells interferes with endothelial cell mobility and decreases expression of VEGF and iNOS, which might result in reduction of ACCs' angiogenesis ability.

In summary, our study shows that inhibition of NF- κ B signalling not only suppresses VEGF and iNOS expression, but also affects endothelial cell mobility. The present data further confirm that NF- κ B and its down-stream factors, VEGF and iNOS, compose a possible signalling pathway in the ACC angiogenesis process, and thus might be a promising anti-angiogenesis strategy in ACC therapy. This significant findings regarding NF- κ B blockage effect on *in vivo* ACC angiogenesis should be thoroughly investigated in future studies.

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