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Advances in Brief

Blockade of Nuclear Factor-κB Signaling Inhibits Angiogenesis and Tumorigenicity of Human Ovarian Cancer Cells by Suppressing Expression of Vascular Endothelial Growth Factor and Interleukin 8[1]

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Abstract

We determined whether blockade of nuclear factor (NF)-κB/relA activity in human ovarian cancer cells can suppress angiogenesis and growth in an orthotopic nude mouse model. The human ovarian cancer cells SKOV3ip.1 and HEY-A8 were transfected with a mutated IκBα (IκBαM), *i.e.*, resistant to phosphorylation and degradation, and hence blocks NF-κB activity. NF-κB signaling blockade significantly inhibited *in vitro* and *in vivo* expression of two major proangiogenic molecules, vascular endothelial growth factor and interleukin 8, in cultured cells and in cells implanted into the peritoneal cavity of nude mice. The decreased expression of vascular endothelial growth factor and interleukin 8 directly correlated with decreased tumorigenicity, decreased vascularization of lesions, decreased formation of malignant ascites, and prolonged survival of mice. These findings suggest that inhibition of NF-κB/relA activity in ovarian cancer cells can suppress angiogenesis and progressive growth.

Introduction

More than 23,000 new cases of ovarian cancer will be diagnosed in the United States in the year 2000 (1), and the mortality rate will be ~50%, making it the leading cause of death from gynecological cancer (1, 2). In most patients, metastasis occurs within the peritoneum by the time of diagnosis. The most common route of spread is by direct extension, and metastatic lesions may develop on any peritoneal surface, including the omentum (2–4). The lesions can give rise to malignant ascites, which inversely correlate with survival (1–4).

Efforts to reduce the growth and spread of neoplasms such as ovarian cancer have focused recently on angiogenesis because they are dependent in part on the formation of adequate vascular support (5, 6). Early studies have demonstrated a complex molecular interplay underlying angiogenesis (5–10). VEGF[3], also known as VPF, has been shown to induce the proliferation of endothelial cells, increase vascular permeability, induce the production of plasminogen activator by these cells, and prolong their survival (7, 8). IL-8, a chemoattractant cytokine, has been shown to attract and activate neutrophils in inflammatory regions and to be angiogenic (9, 10). As is true for other solid malignancies (5), the extent of angiogenesis correlates inversely with prognosis in patients with ovarian cancer (11). Moreover, from studies in our laboratory, we concluded that the expression level of VEGF/VPF and IL-8 by human ovarian cancer cells directly correlates with aggressive disease subsequent to i.p. implantation (12).

The finer details of this interaction are just now becoming understood. Recent studies have demonstrated that the pleiotropic transcription factor NF-κB plays an important role in the control of cell proliferation and apoptosis and hence oncogenesis (13–16). Different neoplasms have been shown to express high levels of NF-κB/relA (17–19). NF-κB/relA has also been shown to regulate the expression of proangiogenic molecules, such as IL-8 (9); however, its role in tumor angiogenesis remains unclear. NF-κB is an inducible dimeric transcription factor that belongs to the Rel/NF-κB family of transcription factors, the prototype of which in most nonlymphoid cells is a heterodimer composed of the RelA (p65) and NF-κB1 (p50) subunits (16). NF-κB complexes are typically retained in the cytoplasm by inhibitory IκB proteins, including IκBα (16). Upon stimulation, IκBα is rapidly phosphorylated and degraded via the ubiquitin-proteasome pathway, permitting activation and nuclear import of NF-κB. Dominant-negative mutant forms of IκBα have been engineered that cannot be phosphorylated and degraded and thus prevent the activation of NF-κB (14–16).

In this study, we set out to determine whether the expression of NF-κB/relA correlates with the degree of neoplastic angiogenesis and with disease progression of human ovarian cancer cells implanted into the peritoneal cavity of nude mice. We show that transfection of malignant ovarian cancer cells with IκBαM, a mutated form of IκBα, decreased the expression of VEGF and IL-8 and, hence, angiogenesis and tumorigenicity.

Materials and Methods

Human Ovarian Cancer Cells. The SKOV3 cell line (12) was originally obtained from the American Type Culture Collection (Rockville, MD). The SKOV3ip.1 variant was derived from ascites arising in a nude mouse injected i.p. with SKOV3 cells (12). HEY-A8 was obtained from Drs. R. Bast and G. B. Mills (Division of Medicine, M. D. Anderson Cancer Center, Houston, TX).

Animals. Female athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 8 weeks of age. Animals were maintained according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

ELISA for Human IL-8 and VEGF Expression. The level of IL-8 protein in culture supernatants was determined by using a quantitative immunometric sandwich enzyme immunoassay (ELISA) kit (Quantikine IL-8 and VEGF ELISA kits; R&D Systems, Minneapolis, MN). The absorbance of the samples was compared with the standard curve (12).

Northern Blot Analysis. Cellular mRNA was extracted from ovarian cancer cells by using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA (2 μg) was separated electrophoretically on 1% denaturing formaldehyde agarose gel, transferred to a GeneScreen nylon

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; NF, nuclear factor; IκBαM, mutated IκBα; IL, interleukin; EMSA, electrophoretic mobility shift assay.

membrane (DuPont Co., Boston, MA) in 20× SSC, and cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). The cDNA probe used in the analysis was a 0.5-kb *Eco*RI cDNA fragment corresponding to human IL-8 and a 0.204-kb *Bam*HI/*Eco*RI cDNA fragment corresponding to human VEGF/VPF (from Dr. B. Berse, Harvard Medical School, Boston, MA). The cDNA probes were labeled with [^{32}P]deoxycytidine triphosphate using a random labeling kit (Roche, Indianapolis, IN). The equivalence of mRNA sample loading was monitored by hybridizing the same membrane filter with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe (20).

Promoter Reporters and Dual Luciferase Assays. Luciferase reporters driven by either two-copy wild-type (2× NF-κB-Luc) or mutant (2× NF-κB-mt-Luc) NF-κB-responsive elements (16) were used in this study. The pGL2-IL8 is a pGL2-basic reporter containing a full-length firefly luciferase gene under the control of an IL-8 promoter flanking from +44 to −1481 from pxp2-IL8 (9). The pGL2-VEGF is a pGL2-basic reporter containing a full-length firefly luciferase gene under the control of both VEGF 5′-flanking region from +50 to −2274 and 3′-untranslated region from 1 to 1921 (21). Ovarian cancer cells (1×10^5/well) growing in six-well tissue culture plates were transfected with 2 μg of the indicated reporter plasmids using the Lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD). Normalization of transfection efficiency was done by cotransfection with 0.05 μg of pB-Actin-RL reporter containing a full-length *Renilla* luciferase gene (Promega Corp., Madison, WI) under the control of the human β-actin promoter (21). Forty-eight h after transfection, the cells were harvested in passive lysis buffer (Promega Corp.). As reported previously, the activities of firefly luciferase and *Renilla* luciferase were quantified using the dual luciferase assay system (Promega). Fold increase of luciferase activity was calculated by comparing test samples to luciferase activity of pGL2-basic in SKOV3ip.1 cells assigned the value of 1 (21).

Stable Transfection of Ovarian Cancer Cells with IκBαM and Control Vector. SKOV3ip.1 and Hey-A8 cells (1×10^6) were transfected using 15 μl of lipofectin reagent (Life Technologies, Inc.) and 4 μg of pLXSN-IκBαM expression vector containing IκBα cDNA with mutations at S32 and S36 of the NH_2 terminus and a COOH-terminal PEST sequence mutation (19) or control pLXSN vector according to the manufacturer's instructions. Cells were selected with standard medium containing 500 and 800 μg/ml G418, respectively. Fourteen days later, Neo-resistant colonies were isolated by trypsinization and established as subcultures. The expression of exogenous IκBαM was verified by Western blot analysis.

Western Blot Analysis. Cytosolic protein was isolated from control and transfected ovarian cancer cells. The soluble protein was separated on 10% SDS-PAGE by electrophoresis and electrophoretically transferred onto Immobilon-P transfer membrane (Millipore, Bedford, MA). The endogenous and mutant IκBα were probed with a polyclonal rabbit antihuman and antimouse IκBα (C-21; Santa Cruz Biotechnology, Santa Cruz, CA). The probe proteins were detected with the Amersham ECL system (Arlington Heights, IL) according to the manufacturer's instructions.

EMSA. Nuclear protein extracts were prepared as described previously (20). The sequence of the NF-κB oligonucleotide probe was 5′-AGTTGAGGGACTTTCCCAGGC-3′. EMSA was performed as described previously (20) with minor modifications. Five μg of nuclear extract protein and 30,000 cpm of end-labeled double-stranded DNA probe were added to the mixture. The binding reaction was allowed to proceed for 25 min at 22°C. For supershift reactions, extracts were preincubated with anti-p65 or anti-p50 antibody (Calbiochem, San Diego, CA) for 20 min on ice.

***In Vivo* Tumor Growth.** Cultured cells were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. A single-cell suspension of 1×10^6 cells with a viability of >95% (trypan blue exclusion) was injected into the peritoneal cavity of female nude mice. The mice were monitored daily for evidence of disease (abdominal swelling, hunched posture, and listlessness) and killed when moribund or 90 days after the i.p. injection. All mice were necropsied, and the pattern (discrete solid lesions, ascites) and extent of abdominal disease (size and number of lesions, volume of ascites) were recorded.

Immunohistochemistry and Quantitation of Microvessel Density. Peritoneal tumors harvested at autopsy were processed for immunostaining using rabbit polyclonal anti-IL-8 (Biosource International, Camarillo, CA), anti-VEGF (Santa Cruz Biotechnology), and anti-CD31/PECAM-1 (PharMingen, San Diego, CA) antibodies and appropriate peroxidase-conjugated antirabbit IgG second antibody. The slides were examined in a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Negative controls were done using nonspecific IgG. The number of blood vessels was counted in each field of each sample (12).

Statistics. The significance of the *in vitro* results was determined by the Student's *t* test (two-tailed), and the significance of the *in vivo* data were determined by the Mann-Whitney *U* test.

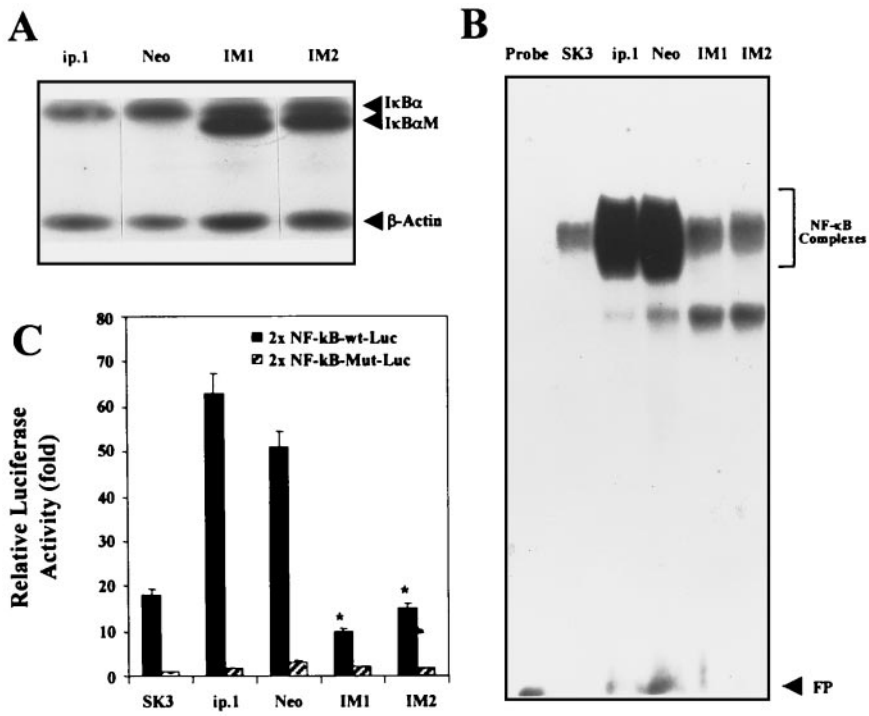

Fig. 1. IκBαM transfection and NF-κB activity. *A*, exogenous IκBαM expression. Cytosolic protein was extracted from SKOV3ip.1 cells (*ip.1*), SKOV3ip.1 cells transfected with pLXSN (*Neo*), and SKOV3ip.1 cells transfected with pLXSN-IκBαM (*IM1* and *IM2*). A Western blot performed using specific anti-IκBα antibody detected endogenous IκBα (higher molecular weight) and exogenous IκBαM (lower molecular weight; *arrows*). Equal loading was monitored by hybridizing the filter with an anti-β-actin antibody. *B*, NF-κB binding activities. Nuclear protein was extracted from SKOV3 (*SK*), SKOV3ip.1 (*ip.1*), SKOV3ip.1 transfected with pLXSN (*Neo*), and SKOV3ip.1 transfected with pLXSN-IκBαM (*IM1* and *IM2*) cells. EMSA was performed using probe only (*Probe*) without nuclear protein as a control. *C*, 2× NF-κB-Luc reporter activities. Luciferase reporters driven by either wild-type (2× NF-κB-wt-luc) or mutant (2× NF-κB-mt-luc) NF-κB response elements were cotransfected with pBActin-RL into the ovarian cancer cells listed above. Firefly and *Renilla* luciferase activities were quantified using the dual-luciferase reporter assay system. Luciferase activity in test samples was compared with the activity in SKOV3ip.1 cells transfected with 2× NF-κB-mt-luc, which was assigned the value of 1. This is one representative experiment of two; *bars*, SD. Note that IκBαM transfection suppressed NF-κB activities (*, $P < 0.01$).

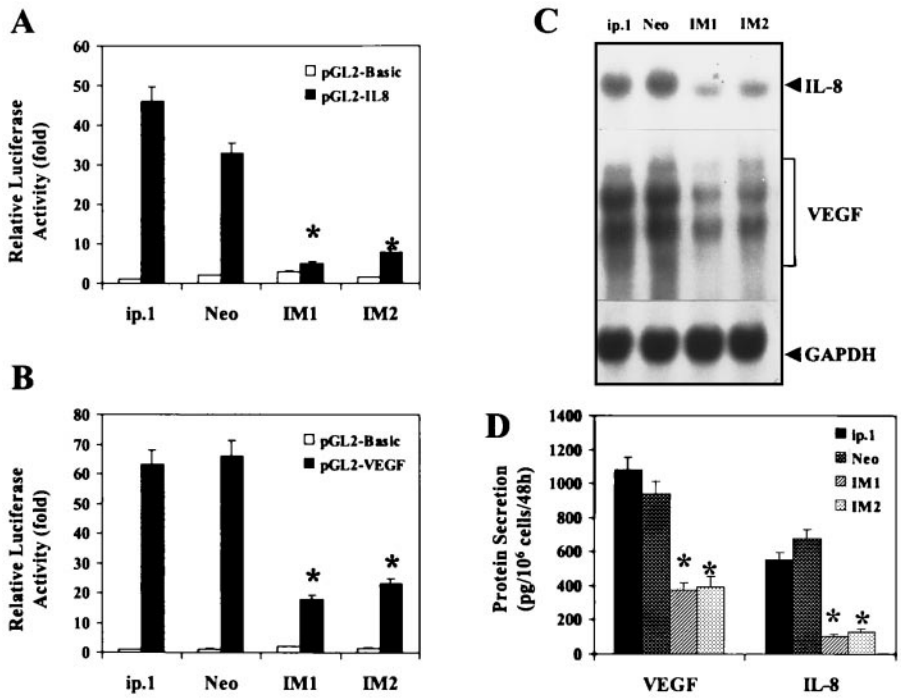

Fig. 2. Expression of *VEGF* and *IL-8* genes. *A*, IL-8 promoter activity. PGL2-IL-8 or pGL2-basic luciferase reporters were cotransfected with pB-Actin-RL (*Renilla*) reporter into the ovarian cancer cells. Luciferase activity was determined as described in Fig. 1*C*. Fold increase in luciferase activity was calculated relative to the luciferase activity of pGL2-basic in SKOV3ip.1 cells assigned the value of 1. *B*, VEGF promoter activity. PGL2-VEGF or pGL2-basic luciferase reporters were cotransfected with pB-Actin-RL (*Renilla*) reporter into the ovarian cancer cells. Fold increase in luciferase activity was calculated as described above. *Bars*, SD. *C*, Northern blot. Cellular mRNA was extracted, Northern blot analysis was performed using IL-8 and VEGF cDNA probes, and equal loading of mRNA was monitored by hybridizing the same membrane filter with a β-actin cDNA probe. *D*, ELISA. IL-8 and VEGF proteins in the culture supernatants determined by ELISA was expressed as pg/10^6 cells/48 h. Note that IκBαM transfection suppressed IL-8 and VEGF protein production (*, $P < 0.01$). *Bars*, SD.

Results

In the first set of experiments, SKOV3ip.1 cells were transfected with the mutated IκBα expression vector (IκBαM). The expression of both endogenous and mutant IκBα was verified by Western blot analysis. As shown in Fig. 1*A*, endogenous IκBα was detected in the parental (ip.1), control pLXSN-transfected (Neo), and IκBαM-transfected cells (IM1 and IM2). Exogenous mutant IκBα was detected only in IκBαM-transfected IM1 and IM2 cells.

To determine the constitutive NF-κB/relA binding activity in the SKOV3ip.1 cells and the effect of IκBαM transfection on NF-κB/relA activity, nuclear protein was extracted from SKOV3 (SK3) cells, SKOV3ip.1 (ip.1) cells, SKOV3ip.1 cells transfected with the control pLXSN vector (Neo), and the SKOV3ip.1 cells transfected with the IκBαM-expression vector (IM1 and IM2). As shown in Fig. 1*B*, low and high constitutive NF-κB binding activity was detected by EMSA in SKOV3 cells and SKOV3ip.1 cells, respectively. The expression of IκBαM significantly inhibited the NF-κB activity in IM1 and IM2 cells as compared with control transfected cells. The specificity of the observed bandshift, checked by supershift experiments with anti-p65 and anti-p50 antibodies, indicated that NF-κB complexes contained both p50 and p65 components (data not shown).

Next, the suppressive effect of IκBαM transfection on constitutive levels of NF-κB/relA activity was next confirmed by NF-κB reporter activity assay. A 2× NF-κB-Luc reporter or a 2× NF-κB-mut-luc reporter (16) was transiently transfected into the SKOV3 variant cells. As shown in Fig. 1*C*, constitutive NF-κB reporter activity was lower in SKOV3 cells as compared with SKOV3ip.1 cells. Moreover, the NF-κB reporter activity was significantly decreased in the IκBαM-transfected IM1 and IM2 cells, which was consistent with the EMSA results (Fig. 1*B*), implying that the SKOV3ip.1 cells constitutively expressed NF-κB/relA activity that could be inhibited by transfection of IκBαM expression vector.

To provide direct evidence for the contribution of NF-κB/relA to the regulation of proangiogenic molecules and hence angiogenesis

Table 1 *Tumorigenicity, vascularization, production of ascites, and survival of nude mice given peritoneal injections of IκB αM-transfected human ovarian cancer cells*

One million viable cells were injected into the peritoneal cavity of female nude mice ($n = 10$). The mice were killed when they became moribund or 90 days after tumor cell injection. The volume of malignant ascites was measured. Solid tumors in the peritoneal cavity were resected and weighed. Mean vessel density was determined by immunohistochemistry of tumor sections using CD31 antibody.

	Tumorigenicity				
	Solid tumors				
Cells	Incidence[a]	Mean weight ± SD (g)	Ascites Mean volume ± SD (ml)	Vessel density[b] (Mean ± SD)	Survival Median days (range)
SKOV3ip.1	10/10	3.2 ± 0.5	2.3 ± 0.7	78 ± 15	45 (32–53)
SKOV3ip.1-Neo	10/10	2.9 ± 0.4	2.6 ± 0.5	71 ± 16	47 (38–60)
SKOV3ip.1-IM1	4/10	0.6 ± 0.8[c]	0.3 ± 0.7[b]	27 ± 6[c]	90 (69−>90)[b]
SKOV3ip.1-IM2	8/10	1.3 ± 0.7[c]	0.6 ± 0.6[b]	37 ± 8[c]	73 (54−>90)[b]
HEY-A8	10/10	2.7 ± 0.7	NA[d]	111 ± 23	34 (26–42)
HEY-A8-Neo	10/10	3.3 ± 0.5	NA	132 ± 32	30 (21–51)
HEY-A8-IM1	9/10	1.6 ± 0.8[c]	NA	58 ± 14[c]	68 (55−>90)[c]
HEY-A8-IM2	7/10	1.3 ± 1.1[c]	NA	49 ± 10[c]	77 (67−>90)[c]

[a] Mice with i.p. tumor/mice that received injections.
[b] Mean number of vessels ±SD per 10 × 200 fields.
[c] $P < 0.001$, Mann-Whitney U test.
[d] No ascites.

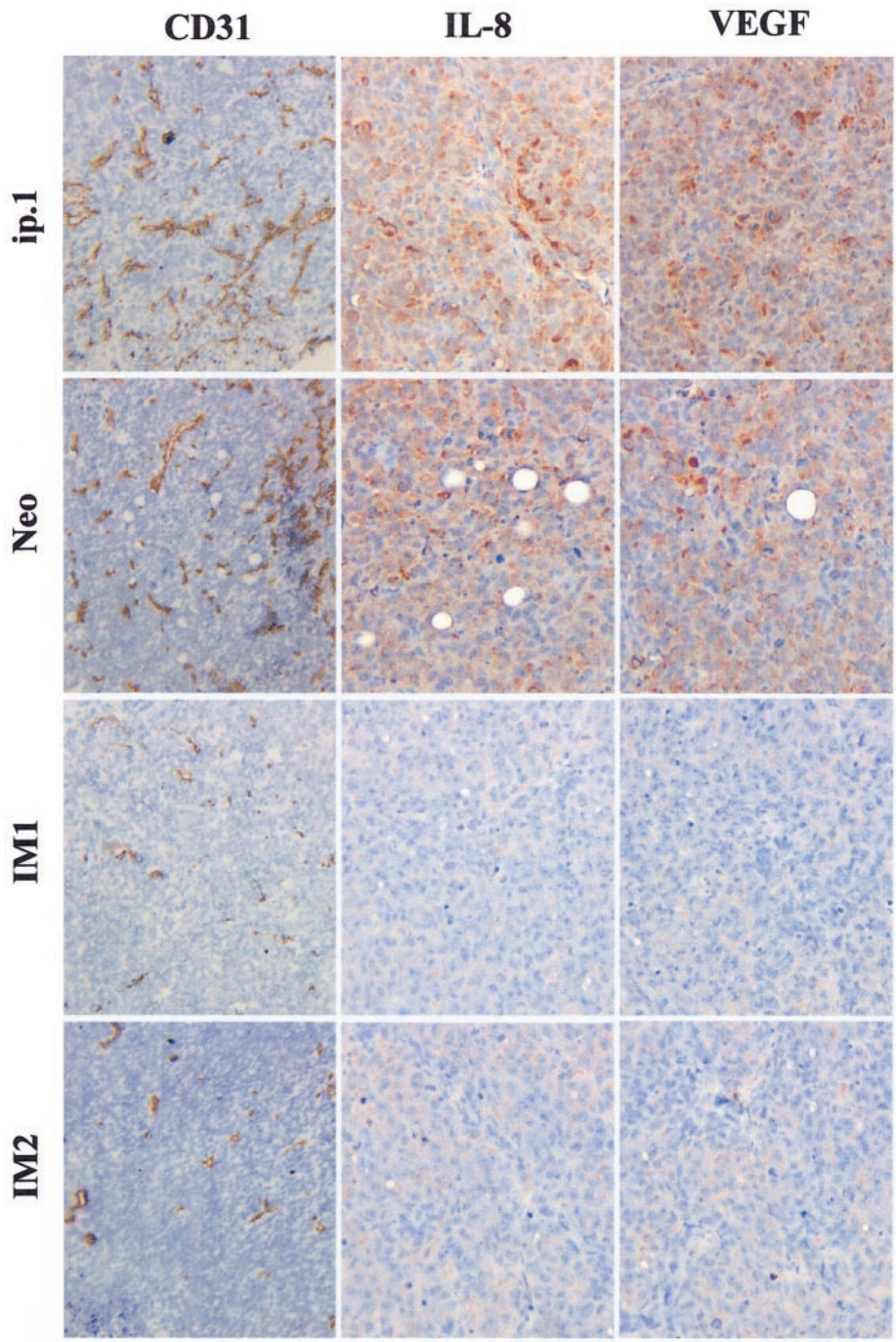

Fig. 3. Immunohistochemistry of ovarian cancers growing in the peritoneum of nude mice. SKOV3ip.1 (*ip.1*) cells, SKOV3ip.1 cells transfected with control pLXSN (*Neo*), and SKOV3ip.1 cells transfected with pLXSN-IκBαM (*IM1* and *IM2*) were injected into the peritoneal cavity of groups of nude mice. Thirty days later, peritoneal tumors with similar size were harvested and processed for immunohistochemical analysis. Blood vessels were counted using a light microscope after immunostaining of tissue sections with anti-CD31 antibodies. VEGF and IL-8 immunostaining was performed as described in "Materials and Methods." Note that tumors formed by IκBαM-transfected cells expressed lower levels of VEGF and IL-8 and were less vascularized than tumors produced by parental or Neo-transfected cells.

and growth of human ovarian cancer, we determined the promoter activities of *VEGF* and *IL-8* genes in the IκBαM-transfected and control ovarian cancer cells. The level of NF-κB promoter activity directly correlated with VEGF and IL-8 promoter activity (Fig. 2, *A* and *B*), suggesting that NF-κB activity regulates VEGF and IL-8 expression at the transcriptional level. The exact NF-κB-responsive elements on the VEGF regulatory regions, however, remain unknown.

Next, the expression of VEGF and IL-8 in IκBαM-transfected cells was determined at both the mRNA and protein levels. Cellular mRNA was extracted from cultured ovarian cancer cells, and Northern blot analysis was performed. As shown in Fig. 2*C*, there was a 3–5-fold decrease in VEGF and IL-8 mRNA expression in the IκBαM-transfected IM1 and IM2 cells as compared with control SKOV3ip.1 and SKOV3ip.1-Neo cells. Consistently, IκBαM-transfected cells secreted significantly decreased levels of VEGF and IL-8 into the culture supernatant, as determined by quantitative IL-8 and VEGF ELISA (Fig. 2*D*). These data show for the first time that NF-κB activity regulates both IL-8 and VEGF constitutive expression in human ovarian cancer cells.

In the next set of *in vivo* experiments, we determined the effect of decreased constitutive NF-κB/relA activity on angiogenesis, tumor growth, and formation of malignant ascites by human ovarian cancer cells. SKOV3ip.1, SKOV3ip.1-Neo, SKOV3ip.1-IM1, and SKOV3ip.1-IM2 cells were injected into the peritoneal cavity of groups of nude mice ($n = 10$). IκBαM transfection inhibited tumor growth and formation of ascites (Table 1). The smaller tumors and low levels of ascites in mice injected with IκBαM-transfected IM1 and IM2 cells correlated with prolonged survival (Table 1). In parallel studies, we also transfected the

human ovarian carcinoma cell line HEY-A8 with the IκBαM expression vector, which also inhibited NF-κB/relA activity and VEGF and IL-8 expression (data not shown). The IκBαM-transfected cells had a significant reduction in tumorigenicity, which in turn correlated with prolonged survival (Table 1).

Finally, we determined whether decreased NF-κB/relA activity and the subsequent decrease in VEGF and IL-8 production by human ovarian cancer cells were associated with suppression of angiogenesis. Tumors produced by control and IκBαM-transfected SKOV3ip.1 or HEY-A8 cells were resected and processed for immunohistochemical analyses of vascular formation using anti-CD31 antibodies. As shown in Table 1 and Fig. 3, tumors produced by control cells were highly vascularized, whereas tumors produced by IκBαM-transfected cells had a significant decrease in microvessel density. Consistent with the alteration of microvessel density, higher levels of VEGF and IL-8 expression were found in tumor lesions produced by control tumor cells than in tumors produced by IκBαM-transfected SKOV3ip.1 cells (Fig. 3).

Discussion

In this study, we correlated the expression level of NF-κB/relA by human ovarian carcinoma cells with disease progression in the peritoneal cavity of nude mice and with angiogenesis. SKOV3 cells produce slow-growing tumors with a low volume of malignant ascites, whereas the SKOV3ip.1 variant (isolated from the SKOV3 parental line) produces rapidly progressing peritoneal disease and a high volume of malignant ascites (12). In agreement with the *in vivo* behavior, the SKOV3ip.1 cells expressed higher levels of IL-8 and VEGF/VPF than did SKOV3 cells (12). Our present data demonstrate that SKOV3ip.1 cells (high expression level of VEGF/VPF and IL-8) expressed significantly higher levels of NF-κB/relA activity than the SKOV3 cells (low expression level of VEGF/VPF and IL-8), suggesting that this transcription factor may play a role in the progression of human ovarian cancers.

Expression of VEGF/VPF and IL-8 by tumor cells directly correlates with angiogenesis (7, 8, 10), which contributes to the progressive growth of neoplasms, including human ovarian cancer (11, 12, 22–24). Specifically, the expression of VEGF/VPF by ovarian cancer cells directly correlates with production of malignant ascites (12, 22). Indeed, the administration of anti-VEGF antibodies to mice injected with human ovarian cancer cells has been shown to inhibit growth of solid peritoneal lesions and formation of ascites (23). The expression level of IL-8 by many human ovarian cancer cells directly correlates with aggressive peritoneal disease (12, 24). However, overexpression of IL-8 achieved by transfection has been shown to retard growth of the transfected cells subsequent to s.c. implantation because of massive infiltration with neutrophils (25). In an orthotopic nude mouse model, however, the constitutive expression of IL-8 by five different human ovarian cancer cell lines directly correlated with disease progression (12), probably by inducing angiogenesis associated with IL-8 interaction with IL-8 receptors on endothelial cells in the peritoneum (10).

The regulation of both VEGF and IL-8 expression during tumor progression may involve diverse mechanisms. Although it is well established that the transcription factor NF-κB is essential for both inducible and constitutive IL-8 expression (9), it is not known whether NF-κB regulates VEGF expression. In this study, we sought to determine whether NF-κB also regulates VEGF expression. IκBαM transfection, which blocks NF-κB activation (19), suppressed the production of both IL-8 and VEGF under *in vitro* and *in vivo* conditions. The significant decrease in VEGF promoter activity found in the IκBαM-transfected cells suggested that the regulation of VEGF by NF-κB probably occurred at the transcriptional level. Studies are under way to define the NF-κB binding site(s) in 5′- and/or 3′-regulatory region(s) of the *VEGF* gene.

NF-κB activation can protect tumor cells from apoptosis; thus, suppression of tumor growth by blocking NF-κB activity could have been attributable to increased apoptosis (14, 15). However, consistent with previous reports showing that stable inhibition of NF-κB in cancer cells by stable transfection of IκBαM does not inhibit cell growth *in vitro* (15, 26, 27), the *in vitro* growth of the IκBαM-transfected SKOV3ip.1 cells was similar to that of control SKOV3ip.1 cells. Thus, the inhibition of tumorigenicity by suppression of NF-κB activity had to occur by other mechanisms, such as inhibition of cell adhesion (28), inhibition of proinflammatory cytokine production (15), or inhibition of plasminogen activator and matrix metalloproteinase (29, 30), which contribute to neoplastic angiogenesis, growth, and metastasis. NF-κB has also been shown to play a role in retinal neovascularization (31) because of its activation by VEGF (32) and in oxidative stress-induced tubular morphogenesis of endothelial cells (33). Our data showing that the NF-κB expression level directly correlates with expression of VEGF/VPF and IL-8 support the role that NF-κB may play in angiogenesis, an essential feature of progressive tumor growth.

In summary, we show that human ovarian cancer cells with high malignant potential express high levels of constitutive NF-κB/relA activity. Suppression of NF-κB/relA activity through expression of a phosphorylation mutant IκBα decreased angiogenesis, retarded tumor growth, and reduced formation of malignant ascites, in part through down-regulation of the angiogenic molecules VEGF and IL-8. These data provide the first direct evidence for the essential role of NF-κB/relA in angiogenesis, tumor growth, and formation of malignant ascites by human ovarian cancer. Targeting NF-κB may therefore be a potential approach to controlling angiogenesis and growth of human ovarian cancer.

Acknowledgments

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References

1. American Cancer Society, Cancer Facts, and Figures. Washington, DC: American Cancer Society, 2000.
2. Ozols, R. F., Schwartz, P. E., and Eifel, P. J. Ovarian cancer, fallopian tube carcinoma and peritoneal carcinoma. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Principles and Practice of Oncology, Ed. 5, pp. 1502–1539. Philadelphia, PA: Lippincott-Raven, 1997.
3. Heintz, A. P. M. Surgery in advanced ovarian carcinoma: is there proof to show the benefit? Eur. J. Oncol., *14:* 91–99, 1988.
4. Roszkowski, P., Wronkowski, J., and Romejko, M. Evaluation of selected prognostic factors in ovarian cancer. Eur. J. Gynecol. Oncol., *14:* 140–145, 1993.
5. Folkman, J. The role of angiogenesis in tumor growth. Semin. Cancer Biol., *3:* 65–71, 1992.
6. Fidler, I. J., and Ellis, L. M. The implications of angiogenesis for the biology and therapy of cancer metastasis (minireview). Cell, *79:* 185–188, 1994.
7. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science (Washington DC), *219:* 983–985, 1983.
8. Leung, D. W., Cachianes, G., Kuang, W. H., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science (Washington DC), *246:* 1306–1309, 1989.
9. Mukaida, N., Okamoto, S., Ishikawa, Y., and Matsushima, K. Molecular mechanism of interleukin-8 gene expression. J. Leukocyte Biol., *56:* 554–581, 1994.
10. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science (Washington DC), *258:* 1798–1801, 1992.
11. Samoszuk, M., Lin, F., Rim, P., and Strathearn, G. New marker for blood vessels in human ovarian and endometrial cancers. Clin. Cancer Res., *2:* 1867–1871, 1996.

12. Yoneda, J., Kuniyasu, H., Crispens, M. A., Price, J. E., Bucana, C. D., and Fidler, I. J. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. J. Natl. Cancer Inst., *90:* 447–454, 1998.
13. Sharma, H. W., and Narayanan, R. The NF-κB transcription factor in oncogenesis. Anticancer Res., *16:* 1589–1596, 1996.
14. Reuther, J. Y., Reuther, G. W., Cortez, D., Pendergast, A. M., and Baldwin, A. S., Jr. A requirement for NF-κB activation in Bcr-Abl-mediated transformation. Gene Dev., *12:* 968–981, 1998.
15. Duffey, D. C., Chen, Z., Dong, G., Ondrey, F. G., Wolf, J. S., Brown, K., Siebenlist, U., and Waes, C. V. Expression of a dominant-negative mutant inhibitor-κBα of NF-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression and tumor growth *in vivo*. Cancer Res., *59:* 3468–3474, 1999.
16. Barnes, P. J., and Karin, M. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases (review). N. Engl. J. Med., *336:* 1066–1071, 1997.
17. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. Mol. Cell. Biol., *17:* 3629–3639, 1997.
18. Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res., *5:* 119–127, 1999.
19. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. Science (Washington DC), *274:* 787–789, 1996.
20. Huang, S., Jean, D., Luca, M., Tainsky, M. A., and Bar-Eli, M. Loss of AP-2 results in downregulation of c-kit and enhancement of melanoma tumorigenicity and metastasis. EMBO J., *17:* 4358–4369, 1998.
21. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J. Biol. Chem., *266:* 11947–11954, 1991.
22. Nagy, J. A., Masse, E. M., Herzberg, K. T., Meyers, M. S., Yeo, K. T., Yeo, T. K., Sioussat, T. M., and Dvorak, H. F. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. Cancer Res., *55:* 360–368, 1995.
23. Kim, J., Li, B., Winer, J., Armaninim, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. Nature (Lond.), *262:* 841–844, 1993.
24. Ivarsson, K., Runesson, E., Sundfeldt, K., Haeger, M., Hedin, L., Janson, P. O., and Brannstrom, M. The chemotactic cytokine interleukin-8–a cyst fluid marker for malignant epithelial ovarian cancer? Gynecol. Oncol., *71:* 420–423, 1998.
25. Lee, L. F., Hellendall, R. P., Wang, Y., Haskill, J. S., Mukaida, N., Matsushima, K., and Ting, J. P. IL-8 reduced tumorigenicity of human ovarian cancer *in vivo* due to neutrophil infiltration. J. Immunol., *164:* 2769–2775, 2000.
26. Bentires-Alj, M., Hellin, A. C., Ameyar, M., Chouaib, S., Merville, M. P., and Bours, V. Stable inhibition of nuclear factor κB in cancer cells does not increase sensitivity to cytotoxic drugs. Cancer Res., *59:* 811–815, 1999.
27. Pajonk, F., Pajonk, K., and McBride, W. H. Inhibition of NF-κB, clonogenicity, and radiosensitivity of human cancer cells. J. Natl. Cancer Inst., *91:* 1956–1960, 1999.
28. Higgins, K. A., Perez, J. R., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A., and Narayanan, R. Antisense inhibition of the p65 subunit of NF-κB blocks tumorigenicity and causes tumor regression. Proc. Natl. Acad. Sci. USA, *90:* 9901–9905, 1993.
29. Wang, W., Abbruzzese, J. L., Evans, D. B., and Chiao, P. J. Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated relA. Oncogene, *18:* 4554–4563, 1999.
30. Bond, M., Fabunmi, R. P., Baker, A. H., and Newby, A. C. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-κB. FEBS Lett., *435:* 29–34, 1998.
31. Yoshida, A., Yoshida, S., Khalil, A. K., Ishibashi, T., and Inomata, H. Role of NF-κB-mediated interleukin-8 expression in intraocular neovascularization. Investig. Ophthalmol. Visual Sci., *39:* 1097–1106, 1998.
32. Marumo, T., Schini-Kerth, V. B., and Busse, R. Vascular endothelial growth factor activates nuclear factor-κB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. Diabetes, *48:* 1131–1137, 1999.
33. Shono, T., Ono, M., Izumi, H., Jimi, S. I., Matsushima, K., Okamoto, T., Kohro, K., and Kuwano, M. Involvement of the transcription factor NF-κB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. Mol. Cell. Biol., *16:* 4231–4239, 1996.

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