Gene Transfer of Inhibitor KappaB in Human Lung Cancer Cell Line NCI-H460 Inhibits Tumorigenesis and Angiogenesis In Vivo

JIAN NI1,2, KOICHI TAKAYAMA1, NAOKO INOSHIMA1, JUNJI UCHINO1, AKIKO HARADA1, TAKAHIRO MINAMI1, TAISHI HARADA1, CAICUN ZHOU2 and YOICHI NAKANISHI1

1Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan; 2Oncology Department, Shanghai Pulmonary Hospital, 507 Zhengmin Rd, Shanghai 200433, China

Abstract. Background: Nuclear factor kappaB (NFÎB) is an inducible and ubiquitously expressed transcription factor which is involved in cell survival, differentiation and growth and, thus, has also been implicated in tumor formation and development. Research on the effect of NFÎB in inhibiting cancer cell growth, however, remains controversial. Materials and Methods: We investigated the effects of overexpressed ÎB· on the proliferation of the human lung cancer cell line H460 in vitro and in vivo using ÎB·-expressing adenovirus. Results: The results suggested that the infection of AdÎB· blocked NFÎB activity in H460 cells and significantly inhibited cell proliferation by inducing apoptosis. An in vivo study showed the tumor incidence to be significantly lower in mice implanted with H460 cells infected with AdÎB·. For established H460 tumor, the intratumoral injection of AdÎB· also inhibited the tumor growth due to both a blockade of the NFÎB activity and an inhibition of the VEGF expression. Conclusion: Adenovirus-mediated ÎB· gene transfer is a promising cancer treatment strategy.

Nuclear factor kappaB (NFÎB) is an inducible and ubiquitously expressed transcription factor. In most untransformed cells, NFÎB is sequestered in the cytoplasm by association with one of its inhibitory molecules, including ÎBÎ, ÎBÎ, ÎBÎ, p105 and p100 and, therefore, NFÎB remains inactive (1). Many stimulators induce the phosphorylation of the ÎB complex and its degradation from the NFÎB-ÎB complex. The liberated NFÎB then translocates to the nucleus where it activates the expression of a series of genes, playing a role in cell survival and functions as an anti-apoptotic factor (1, 2).

Numerous studies have reported a negative correlation between NFÎB expression and cell apoptosis. An anti-apoptotic effect of NFÎB is achieved through its up-regulation of inhibitors of apoptosis (IAPs) and Bel-xl and inhibition of the ensuing activation of caspases (cysteinyl, aspartate-specific proteases) (1). An apoptosis is known to be triggered by the activation of caspases, which include initiators and effectors. To date, there have been no convincing examples of long-term cell rescue once caspases have been activated (3). Caspase 3 is one of the effector caspases, which participates in the apoptotic process of most mammalian cells and activated caspase 3 can only be detected in apoptotic cells (4).

As an important transcription factor in regulating cell growth, the role of NFÎB in tumor formation has gained increasing attention. Many tumor cells derived from non-small cell lung cancer, pancreatic cancer, breast cancer and bladder cancer have been found to express the constitutively activated or increased NFÎB (1). Specimens obtained from patients with cancer also show the existence of a constitutive expression of NFÎB (5). Otherwise, NFÎB plays a role in regulating another key point of tumorigenesis and development, namely, angiogenesis. Fujioka et al. (6) showed the blockade of NFÎB activity in pancreas cancer could inhibit the expression of VEGF and liver metastasis. VEGF functions as a potent pro-survival factor for endothelial cells in newly formed vessels (7). It is expressed by most cancers and is thought to be the most important gene in tumor-associated angiogenesis. In our previous study, we showed suppressed angiogenesis and tumor growth by the gene transfer of a soluble VEGF receptor (8). Because of the potent role of NFÎB in tumor formation and...
development, a lot of studies have been performed to investigate the impact of NF1B inhibition on cancer cells growth. These studies showed the effect to depend on the cell lines evaluated and the different stimulators which activate NF1B (9,10). In addition, the co-expression of some related genes and their products, such as p53 and VEGF, also play an important role in this phenomenon. However, most of these studies have been based on cultivated cells. We thus chose the human lung cancer cell line H460, which is characterized by wild-type p53 and a constitutive activation of NF1B, in the present study. Primarily, we studied the inhibition of NF1B activity on cultivated cells growth and the possible pathway of such inhibition. We next performed a tumor formation test and tumor killing test on nude mice and evaluated the interaction between NF1B and VEGF in this experimental system.

Materials and Methods

Cell culture. A human lung cancer cell line H460 was maintained in RPMI1640 containing 5% FCS and 1% penicillin and streptomycin. The cells were incubated in a moist incubator with 5% CO2 at 37°C.

Viral vectors preparation and transduction protocol. Replication-defective ΔE1 and ΔE3 adenoviral vectors were prepared as previously described (11). A recombinant adenovirus was constructed by in vitro homologous recombination in 293 cells. The desired recombinant adenovirus AdIl Bo (kind gift from Dr.Ueno of the University of Occupational and Environmental Health, Japan) was purified by ultracentrifugation through a CsCl2 gradient and the concentration of vector was decided by tissue culture infectious dose (TCID50). Adenovirus-expressing bacterial β-galactosidase (AdLacZ) was prepared as control. For transfecting, the cells were co-incubated with either AdIl Bo or AdLacZ at varying multiplicities of infection (moi) in a serum-free medium. Two hours later, the serum-free medium was replaced by RPMI1640 containing 5% FCS. The cells were kept in a moist incubator with 5% CO2 at 37°C until used in the experiment.

Gene transfection efficiency assay. 1x10⁶ H460 cells were seeded in a 24-well plate. After 24 hours co-incubation with varying moi of AdLacZ (0-100 moi), the cells were washed and fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 5 minutes at 4°C, and then were stained with β-galactosidase(X-gal). Twenty-four hours later, blue-stained cells were counted with a light microscope.

Western blot analysis. The expression of the transgenic Il Bo was confirmed by a Western blotting assay. After the transfection of AdIl Bo vector at varying moi, cell extracts were prepared by lysing the cells in lysis buffer (10mM HEPES, pH7.5, 3mM MgCl2, 40mM KCl, 2 mM dithiothreitol, 0.5% Nonidet P-40, 8 µg/ml aprotinin, 8 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride) for 15 minutes at 4°C. The soluble protein in the lysates was collected by 20-minute centrifugation at 16,000 rpm at 4°C. The protein concentration was decided by the Bio-Rad protein assay reagent. Next, 20 µl of protein was loaded and separated on 10% SDS-PAGE by electrophoresis. Protein in the gel was transferred onto the hybond-ECL nitrocellulose membranes by a semi-dry method. The membranes were incubated with a rabbit polyclonal anti-AdIl Bo antibody (sc-371, diluted1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and then incubated with a second antibody for 1 hour, at RT. The probed proteins were detected using an ECL Western blotting analysis system (Amersham Pharmacia Biotech, UK).

Cell growth assay. A cell proliferation assay was spectrophotometrically performed using 2-(methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium (Tetra Color One, Seikagaku, Japan). The cells, infected or uninfected with adenovirus, were seeded onto a 96-well plate. After 3 days of culture, the cells in virus-free control wells reached 90% confluence, 10 µl Tetra Color One was added to each well and the absorbance of the cells at 490nm was measured using an ELASA reader (ImmunoMini NJ-2300, InterMed Co., Japan).

Measurement of caspase 3 activity. The cells were incubated with adenovirus and the caspase activity was measured by a fluorometric Caspace Assay System (Promega, Madison, WI, USA), according to the manufacturer’s protocol. Ten µl cell extracts with fluorescent substrate DEVD-AMC were seeded onto a 96-well plate and the fluorescence of cleaved substrates was determined by a spectrofluorometer at an excitation wavelength of 360nm and an emission wavelength of 490nm.

TNFα assay. Cell culture supernatants were collected for TNFα quantitative measurement with a human TNFα ELISA kit (Amersham Pharmacia Biotech, USA) according to the manufacturer’s protocol. Briefly, 50 µl of each sample in duplicate was co-incubated with biotinylated anti-hTNFα antibody and streptavidin HRP conjugate. After removing the unbound conjugate by vigorous washing, a TMB substrate solution was added for color development and the absorbance was measured by an ELISA reader at 450 and 550nm wavelength. The amount of TNFα in the supernatant was determined by interpolating the TNFα concentration from the absorbance of the 450-550nm value using a standard curve.

Tumorogenesis. H460 cells were transfected in vitro at a moi of 30,10 or 3 with AdIl Bo or AdLacZ for 2 days. The cells were recounted, and 6 x 10⁶ viable H460 cells in 100 µl PBS were injected into the flanks of 6-week-old athymic Balb/c nu/nu mice. The experiment was repeated twice and had similar results. Tumor size was measured with a linear caliper every 3 days. Tumor volumes were recorded as the average tumor area (mm²) by measuring the largest perpendicular diameters.

Tumor treatment. H460 cells (6x10⁶) were subcutaneously injected into the flanks of nude mice (6 weeks) on day 0. From day 4, when the tumor volumes were approximately 16 mm², the mice were
injected intratumorally with three increased doses of AdIÎ· or AdLacZ of 30 moi (1.8x10^8PFU), 10 moi (3x10^7PFU) or 3 moi (1.8x10^7PFU) in 100 µl PBS twice a week for 5 times while observing the tumor growth as mentioned above. The mice were sacrificed on day 35 and the xenografts were fixed in 10% formalin for further analysis.

**Immunohistochemistry analysis.** Formalin-fixed, paraffin-embedded xenografts of nude mice were sectioned, deparaffinized in xylene and rehydrated through graded ethanol. The sections were stained with antibodies to P65 and VEGF as described (12,13). Briefly, 1% skimmed-milk in PBS (for p65 stain) or 10% goat serum (for VEGF stain) was placed on the slides to reduce any background staining. The slides were then incubated for 1 hour with anti-p65 rabbit polyclonal antibody (sc-109, diluted 1:50; Santa Cruz Biotechnology) and anti-VEGF rabbit polyclonal antibody (sc-507, diluted 1:50, Santa Cruz Biotechnology) in a moist chamber. The non-specific IgG was used as a negative control. After washing with PBS, the slides were incubated with secondary antibody for 30 minutes (biotinylated anti-rabbit IgG; Nichirei) and then streptavidin-peroxidase reagent (Nichirei) for 30 minutes. The antigen-antibody complex was visualized using a 0.05% solution of diaminobenzidine tetrahydrochloride in PBS. The reactions were quenched by washing with distilled water and then sections were examined after counterstaining with hematoxylin (for VEGF stain). All slides were observed by two investigators (NJ and NI) simultaneously with a double-headed light microscope. P65 expression were evaluated by counting at least 500 cancer cells. A tumor was considered to show a positive stain when the number of cancer cells with stained nuclei exceeded 10% as described by Nishio et al. (14) The VEGF expression was evaluated semiquantitatively according to a method described previously (13,15). The final VEGF expression was scored as a sum of scale a + scale b that reached a maximum score of 6. The scale used herein means the intensity of staining with four levels: negative (score 0), weak (score 1), intermediate (score 2) and strong (score 3). Scale b is the positive cells per 1,000 cancer cells: none (score 0), 1-25% (score 1), 26-50% (score 2) and >50% (score 3).

**Statistical analysis.** Most of the results are shown by the average±mean. The significance of the data was determined by two-tailed Student’s t-test or X² test or Fisher’s exact test as mentioned in the text. A value of p<0.05 was considered to be significant.

**Results**

**Adenovirus-mediated IÎ· overexpression in H460 cells.** To confirm the gene transfer efficiency by adenoviral vector, H460 cells infected with AdLacZ were stained by X-gal and the blue-stained cells were counted. H460 cells were found to be susceptible to adenovirus infection, since 50% of the cells appeared blue with the AdLacZ at 10 moi and 65% at 30 moi (Table 1). The IÎ· expression was confirmed by Western blotting assay. It showed the endogenous IÎ· expression in uninfected cells and significant increase of the expression after IÎ· gene transfer (Figure 1).

**In vitro H460 cell growth inhibition by AdIÎ· infection.** The growth of H460 cells infected with AdIÎ· was inhibited in a dose-dependent manner (Figure 2). A high concentration of adenovirus infection (30 moi) resulted in an inhibition of cell proliferation in both groups (78.5±2.7% in AdIÎ· vs. 89.6±6.6% in AdLacZ, p=0.05). Three moi of AdIÎ· infection caused about 15% cell growth inhibition, while the same dose of AdLacZ infection did not affect the cell
growth. These data indicated that AdI·B· infection at 3 moi induced tumor cell growth inhibition which was independent of the effect of virus infection.

Apoptosis induction in H460 cell by I·B· overexpression. The growth of H460 cells infected with AdI·B· was inhibited in a dose-dependent manner. To confirm that the inhibition was due to apoptosis, we performed double staining with Annexin-V and propidium iodide (PI), which measured the apoptotic cells by an analysis of phosphatidylserine on the outer leaflet of the apoptotic cell membranes. As shown in Figure 3, H460 cells infected with AdI·B· had more annexin stain-positive cells in contrast to the cells infected with the same dose of AdLacZ cells at each moi (p<0.05). This result corresponded to the cell growth assay and indicated that tumor cell apoptosis occurred when NF·B was inhibited. As a result, we adopted 30 moi in the subsequent in vivo study.

Caspase 3 activity in apoptotic H460 cell. Since caspase 3 is the downstream effector caspase in the activation of the apoptosis process and can only be detected in apoptotic cells, we measured the caspase 3 activity in cultivated cells to certify the induction of apoptosis more specifically in this study. As shown in Figure 4, we found an increased caspase 3 activity significantly in H460 cells infected with AdI·B· compared with those infected with AdLacZ at each moi (p<0.05).

TNFa concentration in the supernatant of H460 cells. Caspase 3 is the effector caspase which can be activated by both an intrinsic and extrinsic apoptosis pathway. Death receptors such as TNFa receptor can activate the upstream initiator caspase 8 and induce the intrinsic apoptosis. We, therefore, measured the TNFa concentration in the supernatant of cultivated cells and tried to find a further mechanism of apoptosis in treated H460 cells. The result showed that there was no obvious relationship between the apoptosis and TNFa concentration in treated H460 cells since all samples we tested had almost the same level of TNFa (Figure 5).

In vivo anti-tumor effect by AdI·B· infection. AdI·B· infection induced the apoptosis of H460 cells in vitro, as described above. To clarify whether this strategy is effective in in vivo experiment, inhibition of tumor formation on nude mice was examined. As shown in
Figure 6 and Table II. AdIκBα infection significantly inhibited the H460 tumor formation in comparison to control virus-infected or uninfected tumors. Only two of the nine mice implanted with the AdIκBα 30 moi-infected cells (H460-I30) displayed tumor formation within 6 weeks. In contrast, seven out of eight mice implanted with AdLacZ 30 moi-infected cells (H460-L30) developed a tumor after inoculation (p<0.005). AdIκBα infection at 10 moi marginally inhibited the tumor growth, but not significantly. These results demonstrated that the gene transfer of IκBα inhibited the tumorigenicity of H460 cells in vivo, indicating that NFκB might play a role in H460 tumor formation.

In the next step, we evaluated the anti-tumor effect of AdIκBα for established H460 tumor. Three virus doses, 1.8x10^8 pfu, 3.0x10^7 pfu and 1.8x10^7 pfu of AdIκBα or AdLacZ, were injected intratumorally twice per week. 1.8x10^8 pfu of AdIκBα-suppressed the H460 tumor growth significantly, as shown in Figure 7. No significant difference was seen among the growth curves of the tumors infected with a lesser dose of AdIκBα or AdLacZ. At day 35, these tumors were resected and evaluated for NFκB or related proteins expression immunohistochemically, as described below.

**Immunohistochemical stain for NFκB and VEGF.** We investigated the NFκB expression status in vivo after AdIκBα intratumoral injection by immunohistochemical staining for p65 protein in the resected tumor. The tumor infected with 1.8x10^8 pfu of AdIκBα showed reduced p65 expression immunohistochemically (Figure 8B), while the tumors infected with AdLacZ or mock were positive for p65 (Figure 8A). The data suggested that the overexpressed IκBα suppressed NFκB function in vivo.

**Table II. Tumorigenesis induced by adenovirus infected H460 cells.**

<table>
<thead>
<tr>
<th>H460 cells planted</th>
<th>Tumorigenesis/total planted numbers</th>
</tr>
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<tbody>
<tr>
<td>Uninfected</td>
<td>12/12</td>
</tr>
<tr>
<td>AdIκBα 30 moi-infected</td>
<td>2/9*,#</td>
</tr>
<tr>
<td>AdLacZ 30 moi-infected</td>
<td>7/8</td>
</tr>
<tr>
<td>AdIκBα 10 moi-infected</td>
<td>6/6</td>
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<tr>
<td>AdLacZ 10 moi-infected</td>
<td>6/6</td>
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<tr>
<td>AdIκBα 3 moi-infected</td>
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<tr>
<td>AdLacZ 3 moi-infected</td>
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(The experiment was repeated 2 times with similar results. The data shown here represent the data of two experiments. X2 test, *compared with the uninfected group, p<0.005; #compared with the control virus infected group, p<0.05.)
Discussion

NFκB is involved in most stages of tumor formation and development through modulating such genes and gene products as Bcl-2, Bcl-xl, survivin, cyclinD1, adhesion molecules (AMs), vascular endothelial growth factor (VEGF), MMP-9 etc. (1,2). Inhibiting the activity of NFκB was shown to be an efficient way to control cancer growth and progression in many cell lines (16-18). IκB is well known to inhibit the transactivity of all known NFκB complexes comprising different subunits (19) and is thought to be a useful tool for cancer treatment. In this study, we transferred the IκB gene in human lung cancer cell H460 with recombinant adenovirus, and investigated its effects on cell growth and tumorigenesis. It seemed that NFκB was essential for cell survival and that IκB could inhibit the cell growth directly, since the infected cells had a lower viability than the control virus infected or uninfected culture cells. A reduced cell viability was associated with apoptosis induction. Caspases exist as the apoptotic initiators and effectors, which, once activated, will initiate the death program by destroying key components of the cellular infrastructure and activating factors that mediate damage to the cells (4). Caspase 3 is a kind of downstream caspase and the final effector, which directly mediates the events leading to the death of cells and is thought to participate in most mammalian cells’ apoptosis (20). We found that H460 cells infected with AdIκBα had a dose-dependent increase of caspase 3 activity, thus proving the apoptosis from a molecular level. To elucidate the mechanism of IκBα-induced apoptosis more clearly, we measured the TNFα concentration in the supernatant from culture cells. Though TNFα is thought of as a potent stimulator of tumor cells apoptosis and NFκB is one of the key regulators of TNFα (21), the inhibition of NFκB did not affect the concentration of TNFα in culture cells in this study. From these data, the apoptosis induced by NFκB inhibition does not seem to be related to TNFα.

Since the effect of NFκB is ubiquitous and complicated, cross-talk between tumor-related genes and their products would exist. We performed a tumor formation and tumor treatment in nude mice to observe the effect of gene transfection on tumor growth in vivo.

The effect of NFκB inhibition on tumorigenesis depends on the cell lines evaluated. The studies by Fujioka and his colleagues (6,22), with the same gene transfection method and the same orthotopic nude mice model, and even the same histological type of cancer cells (pancreatic adenocarcinoma cell) but different cell lines (AsPc-1 and Panc-1), showed a different capacity of tumorigenesis. The former could not inhibit, but the latter completely inhibited the tumor formation. In our experiment, tumorigenesis of the human lung cancer cell H460 was significantly inhibited due to the transfer of AdIκBα.

The immunohistochemical staining of the specimens from implanted tumors showed that H460 cancer cells expressed a main subunit of NFκB, p65 in nuclei, thus indicating the
Figure 8. Immunohistochemical staining for p65 and VEGF. A, C, E; H460 tumor injected by AdLacZ was immunostained with anti-p65 antibody (A, x40 magnification) or anti-VEGF antibody (C, x40 and E, x200). Expressed p65 protein and VEGF protein was stained brown. B, D, F; H460 tumor injected by AdIBox was immunostained with anti-p65 antibody (B, x40 magnification) or anti-VEGF antibody (D, x40 and F, x200).
existence of constitutive active NF-\( \kappa B \). Infection with Ad\( \kappa B \) at 30 moi completely eliminated the expression of p65, while 30 moi of AdLacZ and 10 moi of Ad\( \kappa B \) could not inhibit the p65 expression. This proves that the transfection of Ad\( \kappa B \) inhibited the activity of NF-\( \kappa B \) in H460 cells and that this inhibitory effect is linked with the suppression of tumorigenesis.

Even though in culture cells 30 moi of Ad\( \kappa B \) infection inhibited the cell viability to 78.5%, while 30 moi of AdLacZ infection inhibited it to 89.6% (\( p=0.05 \)), and both could induce significant apoptosis, seven out of nine (77.8%) mice transplanted with H460 cells were infected with 30 moi of \( \kappa B \) free of tumor compared with only one out of eight mice (12.5%) implanted with control virus free of tumor (\( p=0.012 \)). This showed that \( \kappa B \) gene transfer inhibited the tumorigenesis not only by directly inducing cancer cells apoptosis, but also by modulating other pathways which were affected by NF-\( \kappa B \) status. Tumor development is known to be a complicated process including cell overproliferation and angiogenesis and increased cell adhesion, as well as immunity of individuals. Especially, angiogenesis is thought to be necessary for tumor growth and metastasis (7). NF-\( \kappa B \) is involved in the control of angiogenesis by directly regulating related genes and cytokines (23,24). VEGF is known to be a most potent factor which can directly activate endothelial cell growth and prompt tumor vessel formation (7). VEGF also plays a role in the immune system by inhibiting the maturation and function of dendritic cells. Patients with non-small cell lung cancer had a poor prognosis when expressing high VEGF and low dendritic cells infiltration (13). Some studies have shown that NF-\( \kappa B \) can regulate the expression of VEGF in multiple stimulators and stress (6). We herein detected the VEGF expression in H460 tumors. As expected, the H460 tumor showed a strong expression of VEGF and the inhibition of activity of NF-\( \kappa B \) significantly reduced this expression. Studies on other cancer cells lines, for example, melanoma A375 cells (25), prostate cancer cell (26), ovarian cancer cell (27) etc., also indicated the effect of the inhibition of NF-\( \kappa B \) on the decreased expression of VEGF and IL-8, thus suggesting that NF-\( \kappa B \) at least partly regulates the expression of angiogenesis-related factors and tumorigenesis and development.

Taken together, the above findings show that transfecting human lung cancer cells with Ad\( \kappa B \) could inhibit the activity of NF-\( \kappa B \), and thereby inhibit tumorigenesis. This is the first report in which \( \kappa B \) gene transfer into the human lung cancer cell line H460 was shown to effectively inhibit tumor formation in an in vivo study. The results showed the effect of transfection to be comprehensive, not only on inhibiting cell proliferation, by inducing cell apoptosis directly, but also by down-regulating the expression of angiogenesis-related genes, e.g. VEGF, to arrest both tumor growth and any potential metastasis.

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References


