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Fundamental principals of tumor necrosis factor-alpha gene therapy approach and implications for patients with lung carcinoma

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Summary Apoptosis, known as programmed cell death, is defined as a cell's preferred form of death under hectic conditions through genetically conserved and complex pathways. There is a decisive balance between stimulatory and inhibitory signaling pathways to maintain homeostasis in cells. In order to shift the balance towards apoptosis, the modulation of both apoptotic and anti-apoptotic pathways represents an attractive target for cancer therapeutics. Currently, chemotherapy and radiotherapy are among the most commonly used treatment modalities against lung cancer. Tumor suppressor gene, p53, is required in order for both of these treatment methods to work as anti-tumor agents. As a result, tumors lacking p53 display resistance to both chemotherapy and radiotherapy. However, death ligands induce apoptosis regardless of p53 status of cells. Thus, these methods constitute a complementary therapeutic approach to currently employed conventional treatment modalities. At present, death ligands are being evaluated as potential cancer therapeutic agents. Since resistance to tumor necrosis factor (TNF)-alpha-mediated apoptosis represented an obstacle for the treatment of patients with lung carcinoma in the earlier attempts, an extensive research was recently initiated to understand molecular mechanism of TNF-alpha signaling. NF-κB transcription factors have been demonstrated to modulate the apoptotic program, mostly as blockers of apoptosis in different cell types. In this review, we concentrate on the current progress in the understanding of TNF-alpha-mediated apoptosis for lung carcinoma. Representative models of NF-κB-inhibiting gene therapy strategies from various labs including ours are also provided as examples of up-to-date approaches to defeat TNF resistance. In order to give the reader better understanding and appreciation of such approaches, previously unpublished in vivo assays are also incorporated into this review. Current progress in clinical trials using adenovirus-mediated delivery of TNF-alpha is also discussed.
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1. Introduction

Lung cancer is still among the most frequently occurring malignancies in the world. 156,900 out of 170,000 patients who develop lung cancer die each year in USA. Although lung cancer is the number one leading cause of death both in men (31%) and women (25%) [1] the death rate from lung cancer in developing countries continues to rise sharply due to smoking habits [2]. Lung cancer can generally be divided into two major histological types as non-small cell lung cancer (NSCLC), which constitutes 80% of all lung cancer cases, and small cell lung cancer (SCLC), which accounts for only 20% of the total. Unfortunately, 5-year survival in patients with NSCLC is only 14%, as opposed to 5—10% in SCLC [3]. Despite the recent advances in chemotherapy, radiotherapy and surgery, survival rates from lung cancer is still very low. Besides, tumors eventually acquire chemo and radio resistance along tumorigenesis. Consequently, other treatment options must be explored in order to get around this problem.

For this reason, gene therapy became the most promising radical treatment modality for lung cancer. One of these gene therapy approaches involves the testing of adenovirus delivery of human TNF-alpha to induce apoptosis in lung cancer cells. TNF-alpha is a well-known cytokine, which is involved in the various cellular activities including cell activation, differentiation and apoptosis [4—6]. However, the cytotoxicity profile of TNF-alpha greatly limited its systemic application for therapy. Hence, a human TNF-alpha mutant (RGD-V29 (F4614)) was generated in order to lower the cytotoxicity profile of TNF-alpha [7,8]. In this study, RGD-V29 exhibited a considerably higher anti-tumor activity against human lung cancer (Mqnu-1) xenografted nude mice without severe organ toxicities, even at the maximal tolerated dose (MTD). On the contrary, severe toxicity at the MTD level was observed when recombinant hTNF was used. Intriguingly, RGD-V29 displayed preferential cytotoxicity toward tumor-associated endothelial cells. For this reason, RGD-V29 is considered to be a mildly toxic mutant with preferential activity towards tumors compared to rhTNF.

In terms of a cellular response; TNF-alpha displays both apoptotic and anti-apoptotic properties depending on the nature of the stimulus and the activation status of certain signaling pathways [9—12]. Under certain circumstances, the up-regulation of NF-κB activity is responsible for the anti-apoptotic effects of TNF-alpha [13,14]. Accordingly, inhibition of TNF-alpha-mediated NF-κB activity represents a potentially useful method for gene therapy by shifting the balance of TNF-alpha stimulation towards cell death [15—17].

2. Molecular mechanism of TNF-alpha-induced apoptosis

TNF-mediated signaling pathways have been studied extensively as summarized in Fig. 1. According to this classical scenario, TRADD is recruited to the TNF-receptor complex as a result of the interaction of TNF with its receptor (TNFR1) [18]. Then, TRADD activates FADD, which is a caspase-8 activator, leading to the initiation of extrinsic and intrinsic apoptotic pathways [19]. TRAF1, TRAF2 and RIP are all recruited to the TNFR1 complex by TRADD [20]. In addition, TRAF1 and TRAF2 have been shown to interact with the anti-apoptotic proteins cIAP-1 and cIAP-2 [21]. Intriguingly, TRADD also stimulates anti-apoptotic NF-κB activity [14,22]. Because of this reason, TRADD is considered to have a dual function: one is prevention of apoptosis through stimulation of NF-κB activity and the other is induction of apoptosis through FADD-dependent pathways. Although the mechanism by which the cell decides for death or survival is not yet clear, according to the current understanding of TNF-alpha signaling, TNFR1-induced apoptosis involves two sequential signaling complexes as shown in Fig. 2 [23]. Complex-I is the initial plasma membrane bound complex, which includes TNFR1, the adaptor TRADD, the kinase RIP1 and TRAF2. Assembling of this complex quickly activates anti-apoptotic NF-κB signaling pathway. On the other hand, complex-II is the cytoplasmic complex, which consists of TRADD, RIP1, FADD and caspase-8. As complex-I activates NF-κB signaling complex, the caspase-8 inhibitor c-Flip (L) is synthesized counteracting complex-II-induced apoptosis and the

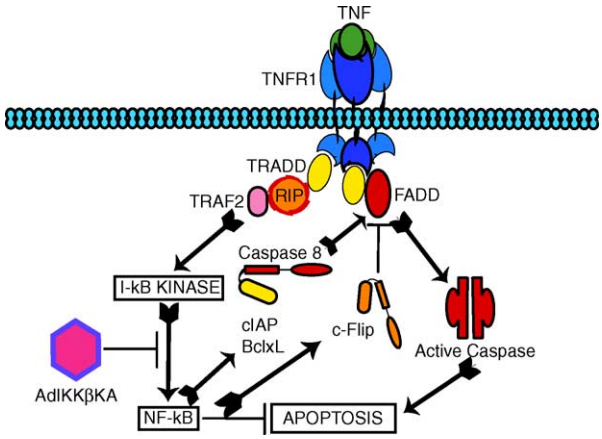

Fig. 1 Classical TNF induced signaling pathway.

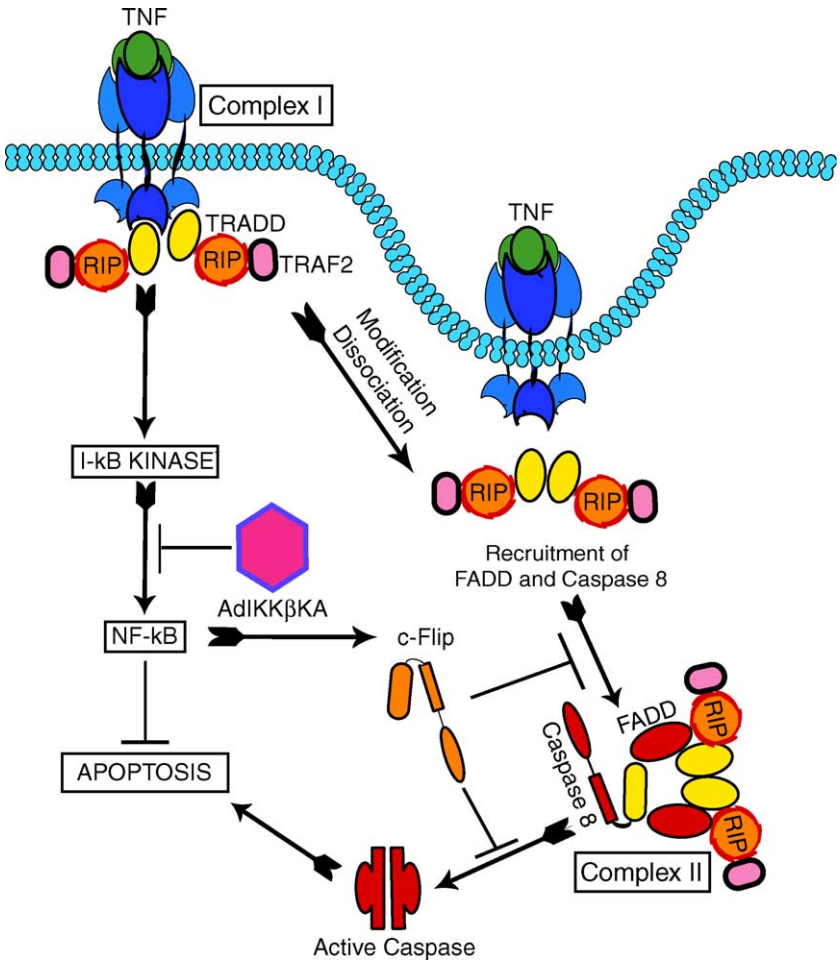

Fig. 2 Current TNF induced signaling pathway.

result is cell survival. These findings suggest that TNFR1-mediated signal transduction cascade possesses a checkpoint in which if complex-I fails to activate NF-κB pathway, activation of complex-II induces cell death. This recent observation also supports our hypothesis that the blocking of NF-κB pathway sensitizes cells to TNF-alpha-induced apoptosis.

3. TNF receptor composition influences the prognosis of patients with lung carcinoma and is the primary requirement for TNF-alpha-induced apoptosis

It has been shown previously by in vitro studies that TNF-alpha exerts anti-proliferative effects on various NSCLCs. However, TNF-alpha administration in patients with advanced NSCLC during clinical trials yielded both conflicting and disappointing results. For this reason, prognostic significance of TNF and TNF receptors in patients with NSCLC was evaluated in detail. To do this, immunohistochemical (IHC) staining was performed on formalin fixed, paraffin-embedded tissues from 39 bronchogenic adenocarcinomas and 32 squamous cell carcinomas using polyclonal antibodies against TNF-alpha, TNF-beta, TNF-R1, and TNF-R2 proteins [24]. The result of IHC analysis suggested that; although NSCLC cells manifested strong co-expression of TNF-alpha, TNF-beta, TNF-R1 and TNF-R2, there appeared to be a loss/down-regulation of TNF receptors in high stage tumors leading to TNF resistance. On the other hand, TNF-R1 and TNF-R2 expression improved the prognosis of patients with NSCLC. These findings suggested that the prognosis of patients with NSCLC and the outcome of TNF-alpha-mediated therapy were mainly determined by the expression pattern of TNF receptors. According to this scenario, TNF receptors would be the primary determinant of TNF-alpha sensitivity in cancer cells. However, despite the expression of 55 kDa TNF receptor (TNF-R55) on the cell surface, the human lung adenocarcinoma cell line, A549,

was still resistant to TNF-alpha-mediated apoptosis. For this reason, the role of two different types of TNF receptors (TNF-R55 and TNF-R75) was investigated in order to find out the mechanism of TNF-alpha resistance by transfecting TNF-R55 or TNF-R75 cDNA into A549 cells [25]. This study demonstrated that TNF-R75 expression mainly provided TNF-alpha-mediated tumor cell lysis in A549 cells, but higher levels of TNF-R55 expression was required to obtain a similar effect.

Additionally, the growth and differentiation effects of TNF-alpha are regulated by retinoids, but the mechanism of this regulation remains to be elucidated. For this purpose, the effects of all-*trans*-retinoic acid (ATRA) on the expression of TNF receptors and receptor-mediated signaling in various human lung cancer cell lines were investigated in detail [26]. It was found that the number of TNF receptors was increased in ATRA treated cells. In addition, the up-regulation of TNF receptor expression sensitized H596 cells to TNF-alpha-induced apoptosis. Intriguingly, A549 cells were naturally resistant to TNF-alpha-induced apoptosis. However, only ATRA treatment converted A549 cells from TNF-alpha resistant to TNF-alpha sensitive phenotype. These results suggested that ATRA up-regulated the expression of TNF receptors in human lung cancer cells. This effect sensitized lung cancer cells to TNF-alpha induced apoptosis.

4. The principle of NF-κB-inhibiting gene therapy strategy to overcome TNF-alpha resistance for lung carcinoma

In resting cells, NF-κB complex consists of two subunits and normally sequestered in the cytoplasm in an inactive state. Upon activation, NF-κB subunits are released from the inhibitory cytoplasmic binding proteins IkB-alpha and IkB-beta and translocate to the nucleus to activate gene transcription. Current paradigms in cancer therapy suggest that activation of NF-κB by a variety of stimuli, including some cyto-reductive agents, may inhibit apoptosis. Thus, inhibiting NF-κB activation may sensitize cells to anticancer therapy, thereby providing a more effective treatment for certain cancers [14]. This idea became the fundamental principle of NF-κB-inhibiting gene therapy approaches currently developed for lung cancer. The purpose was to use viral vectors to deliver genes capable of regulating NF-κB function into the cells in order to sensitize lung cancer cells to TNF-alpha-induced apoptosis. For instance, in order to find out if transfer of the ''super-repressor'' form of the NF-κB inhibitor IkappaBalpha gene could sensitize lung cancer cells to TNF-alpha-mediated cell death, E-1-deleted adenoviral (Ad) vector encoding IkappaBalpha (AdIkappaBalphaSR) was infected into lung cancer cells prior to treatment with TNF-alpha [27]. AdIkappaBalphaSR-mediated gene transfer completely blocked TNF-alpha-mediated nuclear translocation of NF-κB. Although the parental squamous-cell lung cancer cells were resistant to TNF-alpha-mediated cytotoxicity, sensitivity to TNF-alpha was only induced in IkappaBalphaSR-infected cells. These results suggested that IkappaBalpha gene transfer cloned into an adenovirus vector was cytotoxic to squamous-cell lung cancer cells. Targeting other members of NF-κB signaling pathway by gene therapy also reduced TNF-alpha induced NF-κB activity and hence represented potential treatment modality for lung cancer as explained below.

IkB Kinase (IKK) is a protein kinase complex which initiates NF-κB translocation through phosphorylation and degradation of IκB-alpha [28—30]. IKK complex contains two catalytic subunits called IKK-alpha and IKK-beta and a regulatory subunit IKK-gamma [31—35]. Interestingly, mice lacking the IkB Kinase 2 gene (IKK-beta) but not IkB Kinase 1 (IKK-alpha) exhibited severe liver degeneration and this effect was dependent on TNF signaling [36]. Additionally, protection from apoptosis was achieved by the IKK-beta subunit via NF-κB activation in vivo [37]. Because of this reason, an adenoviral construct expressing a dominant negative mutant of IKK-beta (Ad.IKKβKA) was generated in order to investigate the potential of this vector to inhibit NF-κB activation and to promote TNF-alpha-mediated cell death [16,17,38]. Additionally, a dominant negative mutant of Rac1 (N17Rac1) [39] was also tested to see if it could sensitize TNF-alpha induced apoptosis further than what was achieved with Ad.IKKβKA construct alone. Here, we present the summary of such a study including a previously unpublished in vivo observation in support of this hypothesis.

5. A representative model of adenovirus-mediated gene therapy for lung carcinoma in order to increase the therapeutic potential of TNF-alpha in TNF-alpha resistant lung cancer cells

Adenovirus vectors have been tested for delivering therapeutic genes to lung for various purposes. Unfortunately, adenovirus vectors inefficiently transduced normal lung as demonstrated in mice, rats,

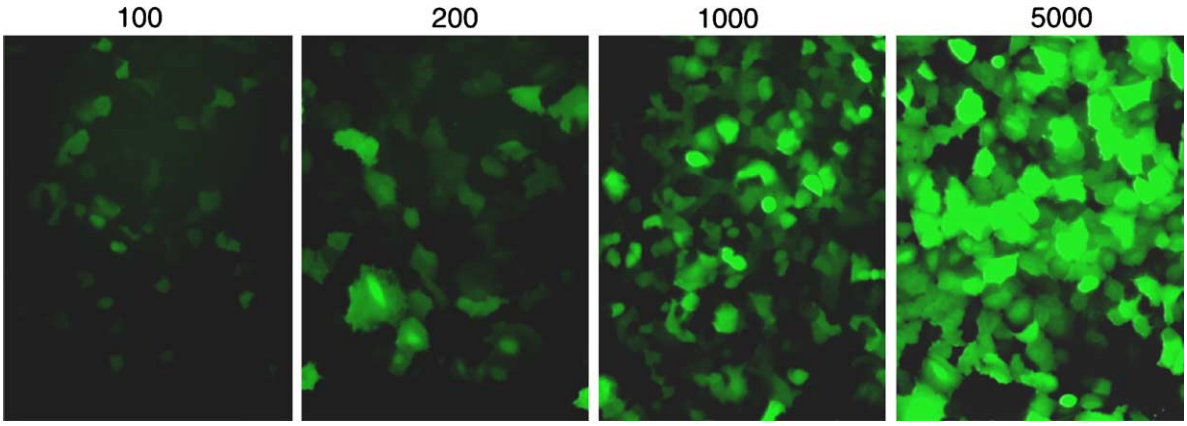

Fig. 3 First generation adenovirus vectors easily transduce lung cancer cells. A549 cells were infected with an adenovirus encoding EGFP reporter gene for 48 h prior to analysis. Then the number of transduced cells is detected under fluorescent microscopy. MOI values for Ad.EGFP expressed as the number of DNA particles/cell of adenovirus are given above the each panel.

primates and humans [40—45]. On the contrary, the transduction of lung carcinoma cells by adenovirus vectors were found to be extremely efficient [46]. Therefore, lung cancer cells represented a good target for adenovirus-mediated gene therapy approach. To confirm this, human lung carcinoma cell line,A549, was infected with an adenovirus encoding enhanced green fluorescein protein (Ad.EGFP) to assess the efficacy of adenovirus infection for lung. More than 90% of cells were transduced with Ad.EGFP virus at an MOI of 5000 DNA particles/cell 48 h following infection (Fig. 3). Furthermore, adenovirus-mediated transduction of lung carcinoma cells appeared to be stable for at least 6 weeks without any toxicity.

In order to find out the effect of NF-κB inhibition on cell survival, A549 cells were first infected with Ad.IKKβKA virus and then treated with hTNF-alpha. Ad.IKKβKA infection followed by hTNF-alpha treatment drastically decreased the cell viability in a dose-dependent manner as shown in Fig. 4. On the other hand, no such effect was observed using Ad.LacZ virus in control experiments. These results suggested that the balance of death or survival signaling by TNF-alpha is modulated by IKKβ in A549 cells. Thus, the susceptibility to TNF-alpha-induced apoptosis appeared to be IKK dependent. In addition, it is well known that apoptotic cell death is induced by caspase activation. In order to further clarify the mechanism underlying the cytotoxic effect, caspase activation assays were performed in A549 cells infected with Ad.IKKβKA virus followed by hTNF-alpha treatment. Only Ad.IKKβKA virus infected-cells manifested caspase-8 activation following hTNF-alpha treatment (Fig. 5). These results suggested that in the setting of NF-κB inhibition provided by the expression of dominant negative IKK-beta, hTNF-alpha activated proapoptotic pathways. Moreover, the expression of a dominant negative Rac1 (N17Rac1) has previously been shown to directly inhibit NF-κB activity leading to sensitization of cells to TNF-alpha-mediated apoptosis [47,48]. In order to determine the effect of a dominant negative Rac1 on cell death, A549 cells were co-infected with both Ad.IKKβKA virus and Ad.N17Rac1 prior to hTNF-alpha treatment. As seen in Fig. 6, approximately 10% cell death was observed with Ad.IKKβKA infection at an MOI of 1000 DNA particles/cell alone followed by TNF-alpha treatment. Co-infection of these cells with Ad.N17Rac1 but not with Ad.LacZ virus yielded a dose-dependent increase in cell death (Fig. 6). These results suggested that inhibiting two different aspects of a converging pathway using both Ad.IKKβKA and Ad.N17Rac1 represented an attractive target for potentially therapeutic interventions targeting lung cancer [16].

To further test the effect of Rac1 and IKK inhibition on cell survival, a mouse model of lung cancer was generated by injecting A549 cells into the flanks of nude mice. Following infection of the tumor cells with recombinant adenovirus vectors containing Ad.LacZ, Ad.IKKβKA, Ad.N17Rac1 or Ad.IKKβKA plus Ad.N17Rac1, the cells were injected subcutaneously into the mice, allowed to grow into tumors, and later harvested for further analysis (Fig. 7). All of the cells were additionally infected with a recombinant adenovirus containing a gene for human TNF-alpha to circumvent species-specific TNF requirements. Our results suggested that both Ad.N17Rac1 and Ad.IKKβKA infections led to a smaller tumor growth compared to Ad.LacZ-infected group or uninfected controls. Co-infection of A549 cells with Ad.N17Rac1 and

Fig. 4 Lung cancer cells are sensitized to TNF-alpha-mediated apoptosis via adenovirus-mediated IKKβKA expression. Ad.IKKβKA virus at indicated MOIs (as shown to the right of each panel) was infected into A549 cells for 48 h. After TNF-alpha treatment (100 ng/ml) for another 24 h, Live/Dead Assay from Molecular Probes was performed. Death cells were stained in red where as live cells were stained in green. Bright field micrographs are also provided for clarity.

Ad.IKKβKA generated the maximum inhibition of tumor growth. In conclusion, the subcutaneous mouse cancer model demonstrated that the combined use of both constructs (Ad.N17Rac1 and Ad.IKKβKA) was more effective in inhibiting tumor growth in nude mice. On the basis of tumor volume, it appeared that IKKβKA expression alone-suppressed tumor growth more effectively than cells infected with Ad.N17Rac1 alone. These results correlated with in vitro experiments where the same MOI of Ad.IKKβKA or Ad.N17Rac1 produced a 95% reduction and a 55% reduction in NF-κB activation respectively (data not shown). Thus, these observations showed that adenovirus vectors are capable of delivering inhibitory genes efficiently, leading to the activation of cell signaling pathways to force cells to undergo apoptosis. Despite the molecular mechanism responsible for linking the Rac 1 pathway to IKK activity is yet unknown, above-mentioned in vivo experiments clearly demonstrated that concurrent inhibition of anti-apoptotic pathways displayed a potential treatment modality for improved tumor cell killing. In these experiments, A549 cells were sensitized to TNF-alpha induced apoptosis by inhibition of Rac1 and IKK pathways and the combinatorial use of Ad.N17Rac1 and Ad.IKKβKA has been proven to be more effective than their individual administrations. Since it would extremely be difficult to infect every single cell in a tumor with adenovirus vectors carrying three different genes through intratumoral injection, this combined approach might be very challenging to employ with

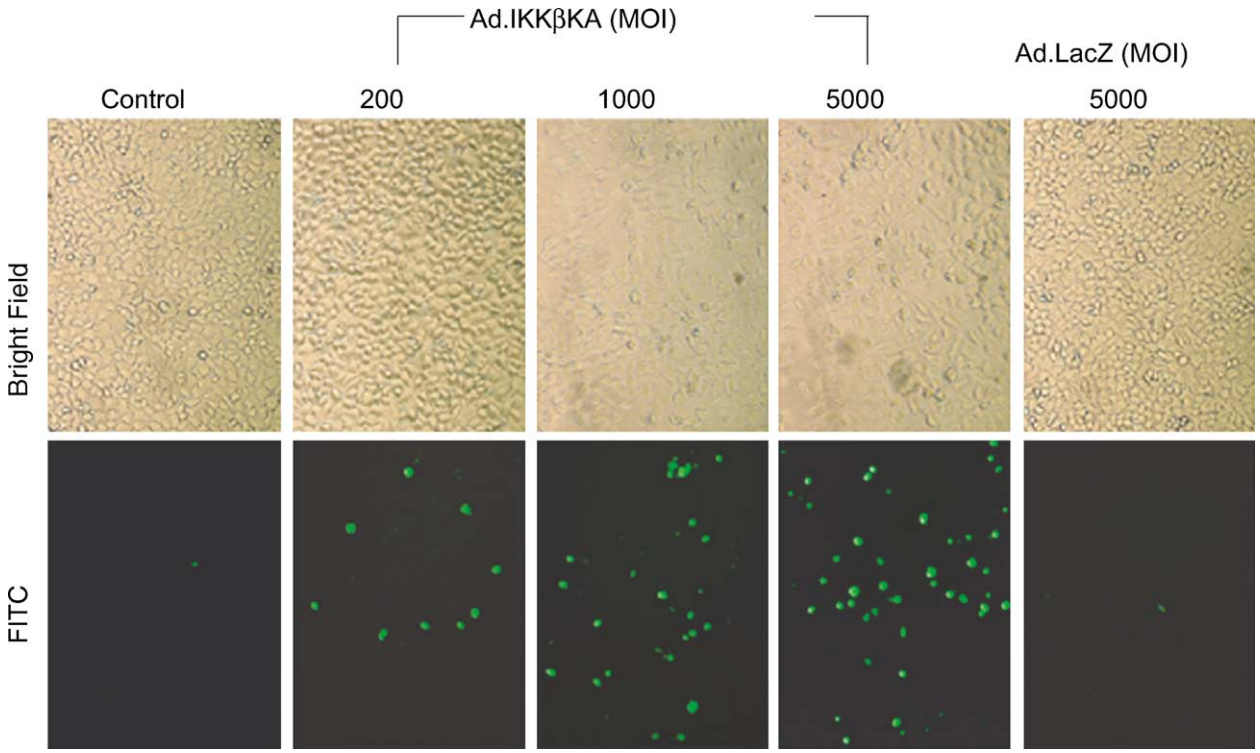

Fig. 5 Adenovirus-mediated IKKβKA expression following TNF-alpha treatment of A549 cells activates caspase pathways. A549 cells were infected with Ad.IKKβKA virus at indicated MOIs above the each panel for 48 h. Prior to caspase-8 activation assay, TNF-alpha treatment was continued for 7 h. Ad.LacZ virus at an MOI of 5000 DNA particles/cell was used as a negative control. In order to selectively assess caspase activation following infection and exposure to hTNF-alpha, CaspaTag™ Caspase Activity Kit (FAM-LETD-FMK inhibitor (S7304)) was used. In this assay, carboxyfluorescein-labeled, caspase inhibitors irreversibly bind to active caspases. Green cells indicate caspase-8 activated cells.

the current technology in a clinical setting for patients with lung carcinoma. Just for the sake of speculation, the following scenario could be tried in a clinical setting. Cancer cells isolated from patients with lung cancer can be transduced with adenovirus encoding TNF-alpha in situ and then these transduced cells can be transferred back into patients in order to boost their immune system. In this clinical setting, however, the outcome of this experiment would have been different than what we observed in animals. Despite cultured lung cancer cells isolated from patients might naturally be resistant to TNF treatment like A549 cell line, TNF treatment alone would be expected to reduce the tumor size in patients with lung carcinoma due to the activation of the patient's cellular immune system. The reason why we did not observe such an effect in animal models is because these animals were nude, lacking effective anti-tumoral T cell response. Nonetheless as explained later in the review, adenovirus vectors, which are currently utilized in clinical trials are much more advanced then the ones we use for animal studies.

6. NF-κB-inhibiting gene therapy strategy overcomes chemoresistance in lung cancer cells

Some lung cancer cells are resistant to chemotherapeutic agents and this is the major reason why some chemotherapeutic approaches fail in lung cancer therapy. In some cases, the activation of NF-κB induced by chemotherapeutic drugs is responsible for the resistance to anticancer drugs. However, the functional role of NF-κB in establishing acquired chemotherapeutic resistance was not studied. For this reason, the ''super-repressor'' form of the NF-κB inhibitor was cloned into an adenoviral vector (ad-IkappaBalpha) and transferred into human lung cancer cell lines, which were resistant to cisplatin (PC-14-DDP) or adriamycin (PC-14-ADR) [49]. Cisplatin and adriamycin resistant lung cancer cell lines (PC-14-DDP and PC-14-ADR) were sensitized to these chemotherapeutic agents by transduction with ad-IkappaBalpha virus. This study confirmed that apoptosis could be induced in these chemoresistant lung cancer cell lines; only if ad-IkappaBalpha gene transfer blocked chemother-

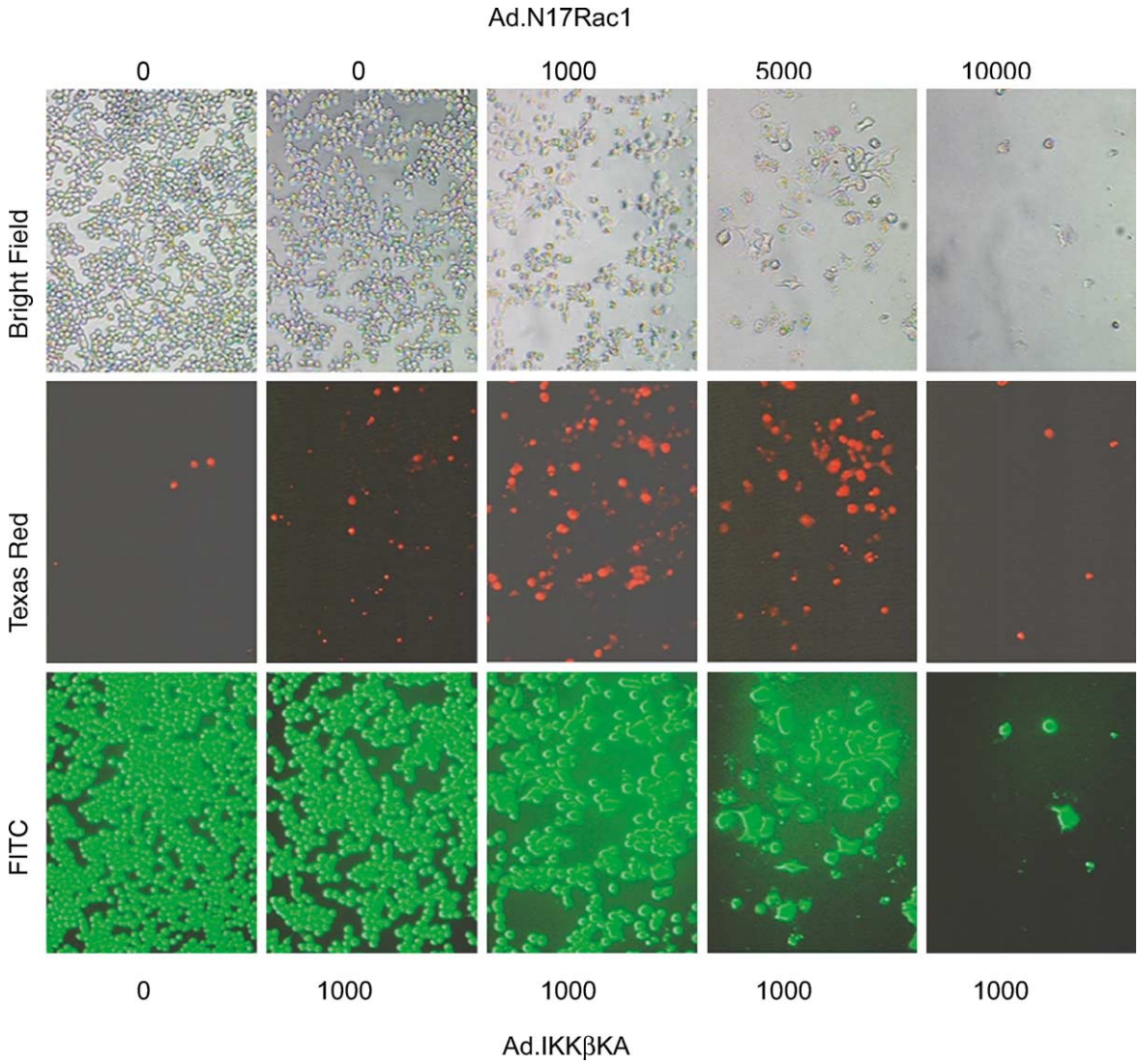

Fig. 6 A549 cells were sensitized to IKKβKA induced TNF-alpha-mediated apoptosis through N17Rac1 expression. Ad.IKKβKA virus was co-infected with Ad.N17Rac1 into A549 cells for 48 h prior to 24 h TNF treatment. Fluorescent photomicrographs of live cells (green) and dead cells (red) in addition to bright fields are shown. MOIs for Ad.N17Rac1 virus are given above each panel. The dose of Ad.IKKβKA virus used for each condition is provided below each panel. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

apeutic agent-induced NF-κB activation. Consequently, adenovirus-mediated IkappaBalpha-SR gene therapy represented a new therapeutic strategy for the treatment of chemoresistant lung carcinoma. In another study, TNF-alpha and several commonly used chemotherapeutic agents up-regulated the expression of anti-apoptotic proteins such as Bcl-x and/or Bfl-1/A1 through an NF-κB-dependent pathway in A549 human lung adenocarcinoma cells [50]. On the other hand, NF-κB-compromised A549 cells were sensitized to both TNF-alpha and chemotherapeutic agents. Differential protections against TNF-alpha and chemotherapeutic treatments were observed by the expression of either Bcl-x or Bfl-1/A1 in the NF-κB-deficient cells. These results revealed a potential strategy to overcome resistance to TNF-alpha or to chemotherapeutic agents in lung cancer cells.

7. Proteasome inhibitors and reactive oxygen species modulate TNF-alpha-induced NF-κB activity

NF-κB activation and nuclear translocation play an important role in preventing apoptotic cell death in some cancers. Consequently, several studies were

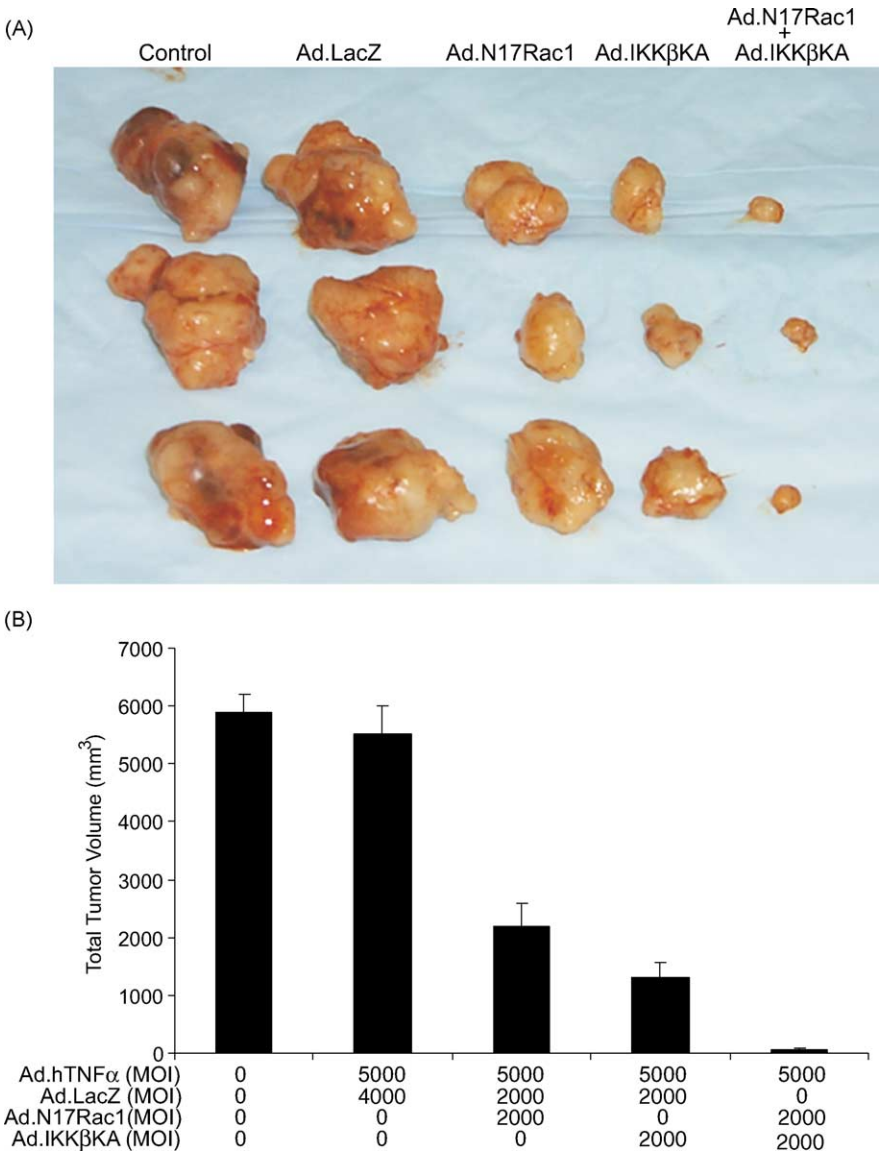

Fig. 7 In vivo testing of the efficiency of N17Rac1 and IKKβKA inhibition of tumor outgrowth. A549 cells were infected with Ad.LacZ, Ad.N17Rac1, Ad.IKKβKA, separately or in combination. All conditions included infection with Ad.hTNFa virus at an MOI of 5000 DNA particles/cell with the exception of the control group. Infected cells were transplanted into the flanks of nude mice. Four mice were used for each group ($n = 4$). Tumor growth was measured 8 weeks after the transplantation. Panel A represents a digital picture of tumor masses excised from these mice. Three out of four examples are given for each condition. Panel B indicates the volume of tumor size in mm as shown on *Y*-axis. The doses of the virus used in the infections are represented on *X*-axis.

carried out in order to investigate NF-κB signaling pathway in non-small cell lung carcinoma. For this purpose, proteasome inhibitors were utilized in order to block NF-κB translocation in human lung adenocarcinoma cells stimulated with TNF-alpha alone or TNF-alpha with proteasome inhibitors [51]. Pre-treatment of lung cancer cells with proteasome inhibitors blocked TNF-alpha induced NF-κB activation. Although TNF-alpha alone was not cytotoxic to cells, proteasome inhibitors mediated the blockade of NF-κB activity and produced TNF-alpha-mediated cell death. In another study, a proteasome inhibitor known as MG132 also blocked NF-κB activation and enhanced TNF-alpha-induced apoptosis in TNF-alpha-resistant lung cancer cell line, NCI-H157 cells [52]. These studies demonstrated that the use of proteasome inhibitors was an effective method of inhibiting NF-κB activity in lung adenocarcinoma cells. Therefore, TNF-alpha treatment in conjunction with proteasome inhibitors yielded a potent stimulus to induce apoptosis.

Recent studies have demonstrated that the superoxide produced within cells act as an intracellular messenger to regulate gene expression in order to modulate cellular activities. The role of active H-ras-mediated superoxide production was studied on tumor cell malignancy in a SV-40 transformed human lung WI-38VA-13 cell line [53]. This study demonstrated that the superoxide induced by ras oncogenic signaling pathway acted as an intracellular messenger to modulate malignant activity such as cellular proliferation, migration and the development of resistance to TNF-alpha. In accordance with this observation, exogenous expression of dominant active form of Rac 1 (V12Rac1) restored resistance to TNF-alpha-induced apoptosis in Ad.IKKβKA-infected A549 cells [16].

8. TNF-alpha and NF-κB signaling regulate metastatic growth of tumor cells

Tumor progression in vivo has been inhibited by forced expression of TNF-alpha in tumor cells through a number of mechanisms involving the activation of the cellular immune system and the induction of apoptosis in target cells. The anti-tumor effects of the TNF-alpha gene transfer were studied in detail using highly-metastatic murine lung carcinoma A11 cells [54]. TNF-alpha-expressing A11 cells were transplanted into syngeneic immunocompetent mice in order to evaluate the effect of TNF-alpha on lung cancer progression. In contrast to mice carrying subcutaneous parental tumors, a significant reduction in the number of spontaneous lung foci formation was observed in mice with A11 tumors expressing TNF-alpha. This suggested that exogenous TNF-alpha expression in cancer cells reduced lung metastasis in vivo. Tumor cell attachment to endothelial cells (ECs) is an essential step in the metastasis of SCLC. Interestingly, TNF-alpha has also been shown to stimulate the attachment of SCLC to ECs [55].

IkappaBbeta, a specific inhibitor of NF-κB, was expressed in human lung adenocarcinoma cell line, A549, in order to evaluate the role of the NF-κB signaling pathway in oncogenic transformation [56]. Anchorage-independent growth was suppressed by transfection of IkappaBbeta into A549 as measured by colony formation in soft agar. Although this approach did not affect in vivo subcutaneous tumor growth in nude mice, the expression of IkappaBbeta drastically inhibited metastatic growth of A549 cells expressing IkappaBbeta in the lungs of nude mice. Based on these observations, it was suggested that NF-κB might play an important role in the anchorage-independent growth as well as the metastatic growth of lung carcinoma cells.

9. TNF-alpha prevents tumor evasion of malignant cells and an essential component of the host defense mechanism

Mononuclear phagocytes produce TNF-alpha cytokine as a defense mechanism against malignant cells. On the other hand, malignant cells can evade this defense mechanism by finding ways to destroy TNF-alpha. Three lung cancer cell lines (small cell carcinoma NCI-H69, adenocarcinoma A-427, squamous carcinoma SK-MES-1) were evaluated in order to delineate the mechanism of tumor evasion against TNF-alpha-mediated host defense [57]. This study indicated that A-427 and SK-MES-1 cell lines produced soluble factors to inhibit TNF-alpha production from monocytes. Indirect immunofluorescence and flow cytometry assays showed that NCI-H69 cells expressed only TNFRI receptor whereas A-427 and SK-MES-1 cells expressed no TNF receptors. For this reason, NCI-H69 and A-427 cell lines displayed resistance to rhTNF-alpha treatment. Consequently, it was concluded that there are various ways for lung cancer cells to evade the host defense. The mechanism of evasion includes the destruction or blocking of TNF-alpha production from monocytes, decreasing TNF receptor expression in cancer cells and blocking the cytotoxic action of TNF-alpha through the alteration of intracellular signaling pathways. Therefore, it is essential to find out the mechanism of tumor evasion before a therapeutic approach can be indicted for lung cancer.

Inhibition of TNF-alpha signaling is not only necessary for sensitizing lung cancer cells to TNF-alpha but also necessary to prevent vascularization of tumors. Vascularization is an important aspect in the growth and metastasis of solid tumors. Vascular endothelial growth factor (VEGF) is claimed to be the most potent and pathologically important among the angiogenic factors produced by tumor cells [58]. In this context, TNF-alpha-stimulated Sp1 activation is thought to be essential for the synthesis of VEGF [59]. Transfection of Sp1 decoy oligodeoxynucleotides decreased metastasis and cellular proliferation of both A549 and U251 cell lines. These results proposed that the Sp1 decoy strategy would be effective in reducing angiogenic growth factor expression, proliferation and invasiveness of tumor cells.

10. Current update on clinical trials using adenovirus-mediated TNF-alpha delivery for tumors

Currently, there are four ongoing clinical trials using TNF-alpha-mediated gene therapy approach for cancer as reported to the *Journal of Gene Medicine* website by the year 2002. All four use viral vectors by means of delivering TNF-alpha gene to patients. Two of these approaches utilize adenovirus vectors and the other two employ retrovirus vectors. Dr. Steven A. Rosenberg from National Cancer Institute, Bethesda, MD, USA, has been conducting two clinical gene therapy approaches involving retrovirus-mediated TNF-alpha gene delivery to patients with cancer. Retrovirus vectors carrying TNF-alpha gene were infected into either tumor infiltrating lymphocytes (TILs) or tumor cells in situ. Then these autologous tumor cells or TILs were injected back into patients in order to boost the immune system. Targeted cancer types in these trials were melanoma, renal cell cancer, colon cancer and breast cancer. First clinical study of adenovirus-mediated TNF delivery is an open-label, phase I, dose-escalation study of TNF-alpha (TNFerade^{TM} Biologic) gene therapy complemented with radiation for locally advanced, recurrent or metastatic solid tumors. This study has been conducted by Dr. Jigna Desai Jhaveri from Albert Einstein College of Medicine, Bronx, New York, USA. TNFerade^{TM} Biologic is a replication-deficient adenovirus gene therapy vector without functional E1, E3 and E4 gene expression. AdEGR.TNF vector was generated by cloning of human TNF-alpha c-DNA into TNFerade vector. Since TNF-alpha acted synergistically with radiation as shown in previous studies, this vector also contained radiation-inducible early growth response elements (Egr-1) ligated upstream of TNF-alpha c-DNA. This allowed maximum expression of TNF-alpha only upon radiation. Previously, a similar vector called Ad5.Egr-TNF was tested on animal models and this vector demonstrated high efficiency of tumor cell killing in addition to a low toxicity profile. No systemic side effects were observed. There were two primary objectives of using TNFerade^{TM} Biologic for clinical trials. The first objective was to investigate the safety and the practicality of intratumoral injection of TNFerade^{TM} Biologic combined with radiation therapy. The second objective was to determine the maximum tolerated dose (MTD) of TNFerade^{TM} Biologic and radiation in patients with locally advanced, metastatic and recurrent solid tumors. Initial results from this trial suggested that the intratumoral administration of TNFerade^{TM} Biologic provided a convenient way of delivering TNF-alpha locally. In addition, anti-tumoral response was significantly augmented upon radiation. The second clinical study using adenovirus-mediated TNF-alpha gene delivery is an open-label, phase I, dose-escalation study of TNFerade^{TM} Biologic with radiation therapy as an adjunct to surgery or for palliation of soft tissue sarcoma of the extremities. Dr. Nader Hanna from University of Kentucky Chandler Medical Center, USA, has been conducting this study, which was sponsored by GenVec. It is interesting to note that the same adenovirus construct (TNFerade^{TM} Biologic) was used in both of these clinical trials. Therefore, TNFerade^{TM} Biologic represents the first gene therapy vector clinically being tested for adenovirus-mediated TNF-alpha gene therapy targeting cancer cells. These clinical trials are still open and clear conclusions will only be reached upon the completion of these studies.

In conclusion, there is a great progress made within the last couple of years in understanding of the molecular mechanism of TNF-alpha-mediated signaling. This has certainly paved the way to new targets. NF-κB signaling pathway, which is primarily responsible for the growth and metastatic nature of tumors, appears to be the main candidate for TNF resistance observed in lung cancer cells. Despite TNF-induced NF-κB activity can be inhibited using proteasome inhibitors, gene delivery strategies targeting this pathway represented attractive models of gene therapy for lung cancer as described in this review. The diversity of gene therapy approaches, which could be designed for the treatment of patients with lung carcinoma, will completely rely on the upcoming advancement in understanding of TNF-alpha-mediated signaling pathway.

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