Involvement of STAT3, NF-κB and associated downstream molecules before and after the onset of urethane induced lung tumors in mouse☆

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ABSTRACT

Here we have shown the alteration of transcription factors STAT3, NF-κB and downstream associated molecules much before the appearance of lung tumor and their response to antitumor agent, inositol hexaphosphate. Histological examination revealed the pathophysiology of the lung tissues and the onset or progression of tumor from 4 or 9 to 24 weeks in terms of tumor volume and the number. Over expression of NF-κB (p50/Rel A), COX-2, STAT3, pSTAT3 (Tyr 705), IL-6 and cyclin D1 also progressed from the time of no tumor to the time of tumor appearance and was reduced in mice drinking 2%IP6. We suggest that the alterations of STAT3, NF-κB and downstream associated molecules are critical in the development of lung tumors and can be exploited as possible mechanisms after the exposure. Status of these altered genes before the tumor development suggests their possible use as targets for the tumor control in the predisposed conditions.

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1. Introduction

Lung cancer is a leading cause of cancer-related deaths all over the world. Non-small cell lung carcinoma (NSCLC) is prevalent and increasing in both smokers and non-smokers because of its late diagnosis and poor prognosis. The better understanding of the mechanics of the development of lung tumor before its appearance could be of use in its control (Herbst et al., 2008). Certain neoplasia forming genetic alterations induces inflammation and predisposes individuals to various types of cancer development. Inflammation is tightly involved in the process of carcinogenesis – from its genesis, progression to advancement of cancer (Mantovani et al., 2008). But there is a lack of

reports showing molecular correlation between the inflammatory pathways as early or late events in tumorigenesis.

Chemical carcinogenesis in mouse lung mimics human lung carcinogenesis in various aspects. Due to the molecular and histological similarities between murine and human adenocarcinoma, urethane induced NSCLCs model in mice is often employed for tumor development and chemopreventive studies (Stathopoulos et al., 2007; Meylan et al., 2009). Therefore, exploring molecular event of tumorigenesis before the appearance of tumor and their correlation with tumor development can give an insight into cancer development and control. Since inflammation precedes cancer, we tried to evaluate whether the molecular changes leading to inflammation

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starts appearing much before the appearance of hyperplasia or tumor.

More than 50% NSCLC primary tumors show persistent STAT3 activation (Gao et al., 2007). Signal transducer and activator of transcription-3 (STAT3) and nuclear factor-κB (NF-κB) are the prime transcriptional molecules (Mantovani et al., 2008) targeting IL-1, IL-6, TNF, cyclin D1, COX-2 etc. (Meylan et al., 2009; Dolcet et al., 2005; Nishikori, 2005). One of the critical molecules involved in inflammation is COX-2, which has also been shown to be frequently altered in various cancer types and cancerous cells (Kisley et al., 2002).

STAT3 is activated by phosphorylation of Tyr at 705 positions at the C terminus of protein. A large body of evidences supports the deregulation of growth and survival, the promotion of angiogenesis, and the suppression of host's immune surveillance of tumor in response to STAT3 activation. Moreover, aberrant STAT3 promotes invasion and metastasis, thereby contributing to tumor progression (Yue and Turkson, 2009). IL-6, a cytokine, has multiple functions and has role in epithelial tumors (Schafer and Brugge, 2007). IL-6 can induce STAT3 pathway for cell survival. Level of pSTAT3 has been correlated with the level of IL-6 in many types of cancers (Gao et al., 2007; Kishimoto, 2005).

NF-κB family of proteins regulates different pathways leading to cell survival and cancer pathogenesis and shown to be activated at early stage of carcinogenesis (Stathopoulos et al., 2007) showing suppressed apoptosis and increased cell survival. The most common NF-κB dimer in mammals contains p50 and RelA (p65) and is found to be activated in many types of cancer (Dolcet et al., 2005; Nishikori, 2005). Though, the impact of STAT3 and NF-κB on lung tumorigenesis with the passage of time has not been investigated, both the pathways enhance cell survival by transcribing cyclin D1 and Bcl family proteins (Jarnicki et al., 2010).

Chemopreventive intervention to any molecular change could give an insight to targeted cancer therapies and prevention. We have used inositol hexaphosphate (IP6), a naturally occurring sugar phosphate, exhibiting tumor suppressing effect through its anti-angiogenic, anti-oxidant and anti inflammatory properties (Gupta et al., 2003; Raina et al., 2008; Vucenik et al., 2004). Urethane, an ethylcarbamate, was used as a tumorigen. Urethane-induced lung tumorigenesis correlates with the development of lung inflammation by activation of NF-κB at early time points (Meylan et al., 2009). Understanding of the representation of inflammatory changes before the appearance of tumors could be helpful in stratification of tumor development and its control.

2. Materials and methods

2.1. Animals

Female Balb/c mice (4–5 weeks old) from the inbred colony of IITR, Lucknow were used throughout the study. Animals were kept in 12 h light and dark cycle, fed on synthetic pellet diet (M/S Ashirwad Pvt. Ltd, Chandigharh, India) and water ad libitum. Animals were handled according to norms of institutional animal ethics committee (IAEC).

2.2. Animal treatment

Animals were randomly divided in 4 groups each consisting of 9 animals. Treatment details are given in Table 1. To hasten the process of tumorigenesis, we administered 7 weekly i.p. injections of urethane (Sigma). Administration of urethane was followed as described (Wang et al., 2009). Selection of IP6 (Sigma) dose is based on earlier reports (Raina et al., 2008; Vucenik et al., 2004). Mice were observed for ill health or mortality regularly. Animals from each group were sacrificed by cervical dislocation at the end of 9 or 24 weeks from the time of first injection of urethane. In a separate study to analyze the state of no tumor, mice were given 3 weekly i.p. injections of urethane and were sacrificed at the end of 4 weeks after the first urethane injection. Lungs from one third of the mice from each group were fixed in 4% buffered formalin for histopathological analysis and lungs from rest of the mice in each group were taken out and processed for the molecular studies in respective groups.

2.3. Histopathology

Formalin fixed lungs were processed as described (Raina et al., 2008). 5 μm H&E stained sections were examined using Leica DFC 295 camera under Leica DM 1000 microscope at the magnification of 100×. Lung tumors were identified as adenoma or carcinoma. Diameter of the largest section of each tumor was measured with Leica live measurement software and used to calculate the area of tumor in mm^2 (Bauer et al., 2000).

2.4. Reverse transcription of RNA (RT-PCR)

RNA was isolated by TRIzol (Invitrogen) and was treated with DNaseI (Ambion) to remove DNA contamination, if any. cDNA, was synthesized by RT-PCR using AMV RT kit (Bangalore Genei, India) and was amplified using mRNA specific primers (MWG Bio Tech, Germany) for specific genes (Table 2). Ampli Taq DNA polymerase (Ambion) was used for PCR with the hot start at 95 °C for 10 min, 35 cycles (95 °C for 60 s, annealing for 60 s and 72 °C for 60 s) was done with a final extension at 72 °C for 4 min (Schafer and Brugge, 2007). PCR products were resolved on 1.5% agarose and ethidium bromide gel and were quantitated using Gene Tool Syngene software.

2.5. Western blotting

As reported (Gao et al., 2007), an aliquot of the lung tissue extract in 20 mM Tris buffer containing sucrose, MgCl$_2$, EDTA, DTT, Na$_3$VO$_4$, PMSF and protease inhibitor cocktail (Sigma) was subjected to SDS-PAGE on 7.5–12.5% Tris–glycine gel. The separated proteins were transferred onto PVDF membrane (Millipore) and were probed with NF-κB1 (p50), Rel A (p65), STAT3, pSTAT3 (Tyr 705), COX-2, IL-6, IκBα or cyclin D1 antibodies (Santa Cruz Biotech) using peroxidase-conjugated appropriate secondary antibody (Bangalore Genei, India). Signals were visualized by Chemilumniscence HRP detection system (Millipore) on Versa Doc (Bio-rad). Membranes were stripped and re-probed with β-actin antibody (Sigma).