Eur Respir J 2006; 28: 651–661
DOI: 10.1183/09031936.06.00012106

SERIES "SIGNALLING AND TRANSCRIPTIONAL REGULATION IN INFLAMMATORY AND IMMUNE CELLS: IMPORTANCE IN LUNG BIOLOGY AND DISEASE"
Edited by K.F. Chung and I.M. Adcock
Number 7 in this Series

c-Jun N-terminal kinase-dependent mechanisms in respiratory disease

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ABSTRACT: Respiratory diseases pose a multifaceted dilemma. Although the symptoms and pathology are obvious and provide multiple opportunities for therapeutic investigation, at the same time, the molecular complexities and prioritisation are overwhelming.

Even within a disease such as asthma, the number of inducers, cell types, secondary mediators, chemical changes, immune responses and tissue modifications is remarkable. One means of therapeutically targeting this complexity is to identify individual factors responsible for regulating multiple disease processes.

The mitogen-activated protein kinase family integrates multiple diverse stimuli, and, in turn, initiates a cell response by phosphorylating and thereby modulating the activity of many target proteins. The c-Jun N-terminal kinase is a critical regulator of pro-inflammatory genes, tissue remodelling and apoptosis, and, therefore, represents an attractive target for novel therapies.

Pre-clinical and clinical investigation into the efficacy of c-Jun N-terminal kinase inhibitors has been ongoing since the late 1990s. Over the course of this work, hypotheses have shifted as to the role of c-Jun N-terminal kinase in the many processes that promote allergic, inflammatory, obstructive and fibrotic diseases of the lung. Inhibition of c-Jun N-terminal kinase may indeed provide a means of suppressing more pathological mechanisms in respiratory disease than first suspected.

KEYWORDS: c-Jun N-terminal kinase, inflammation, kinases, pharmacotherapy, signal transduction

Mitogen-activated protein (MAP) kinases are a conserved family of enzymes that relay external stimuli through the cell using phosphorylation cascades, to generate a coordinated response by the cell to its environment (fig. 1). The three main MAP kinase pathways are named after the terminal kinase in each cascade, extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK).

In *Saccharomyces* and *Drosophila*, MAP kinase homologues are involved in responses as diverse as pheromone binding, osmotic shock and embryo development. In mammals, the role of these pathways is also wide ranging. Additional complexity is introduced with the presence of three ERK, four p38 and three JNK isoforms. Knockout mice have been generated for almost all of the MAP kinase isoforms, and the phenotypes indicate some unique roles for each of these proteins (table 1; [1–14]) [15].

In general, ERK isoforms are critical effectors of growth factor stimulation and tissue development,

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Received:
January 26 2006
Accepted after revision:
March 25 2006

Previous articles in this series: No. 1: Fan J, Heller NM, Gorospe M, Atasoy U, Stellato C. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur Respir J* 2005; 26: 933–947. **No. 2:** Georas SN, Guo J, De Fanis U, Casolaro V. T-helper cell type-2 regulation in allergic disease. *Eur Respir J* 2005; 26: 1119–1137. **No. 3:** Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-β and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 2006; 27: 208–229. **No. 4:** Barnes PJ. Corticosteroid effects on cell signalling. *Eur Respir J* 2006; 27: 413–426. **No. 5:** Giembycz MA, Newton R. Beyond the dogma: novel β_2-adrenoceptor signalling in the airways. *Eur Respir J* 2006; 27: 1286–1306. **No. 6:** Rahman I, Adcock IM. Oxidative stress and redox regulation of lung inflammation in COPD. *Eur Respir J* 2006; 28: 219–242.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

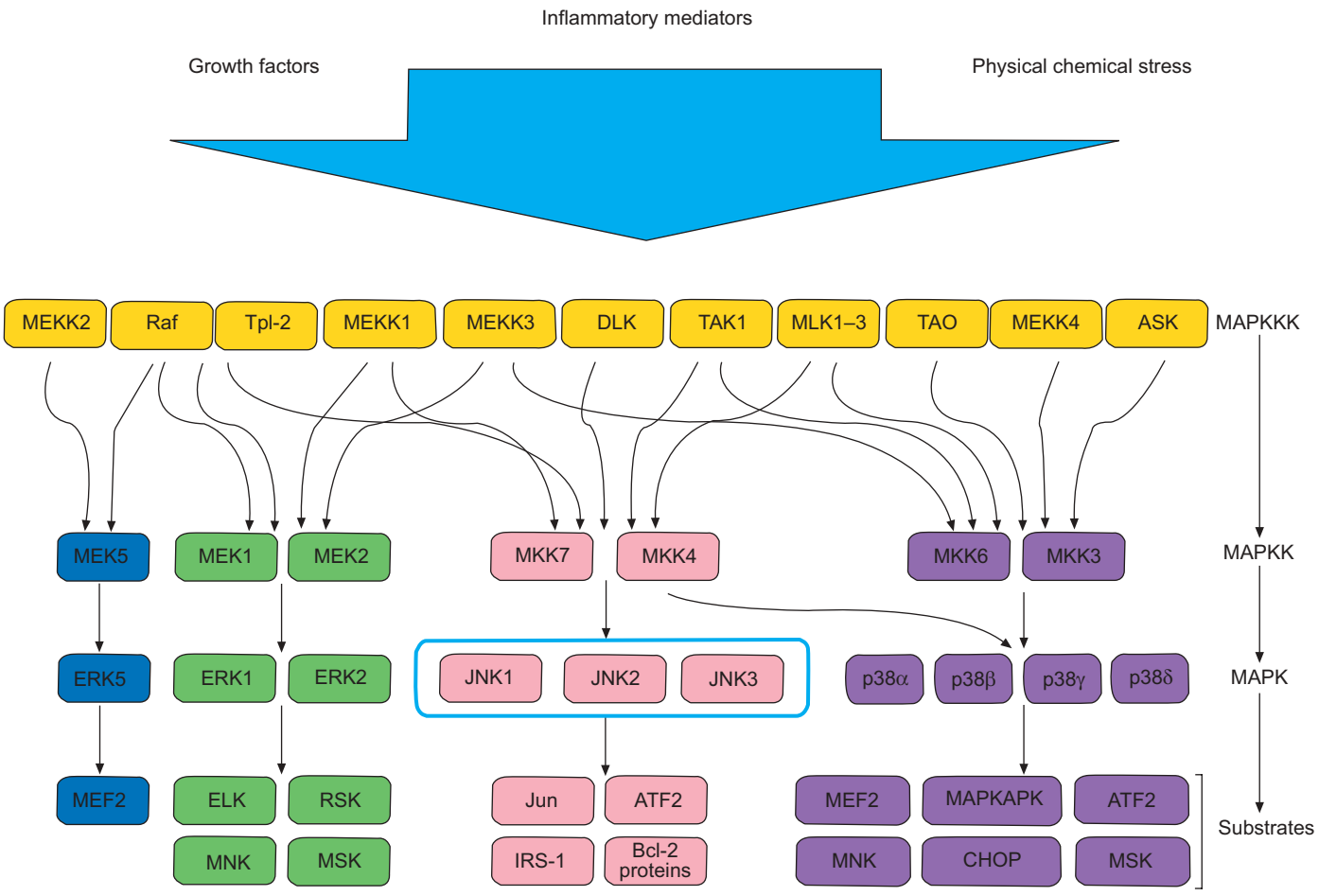

FIGURE 1. Mitogen-activated protein (MAP) kinase (MAPK) signalling cascades in mammalian cells: a schematic summary of the generally accepted interactions between protein kinases of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 pathways. Unique interactions may exist in certain cell types and with specific stimuli. Not all proposed MAPK substrates are shown. The box highlights the location of JNK in the signalling network. MAPKK: MAP kinase kinase; MAPKKK: MAP kinase kinase kinase; MEK: MAP/ERK kinase; MEKK: MAP/ERK kinase kinase; Tpl-2: mitogen-activated protein kinase kinase kinase 8; DLK: dual leucine zipper-bearing kinase; TAK: transforming growth factor-β-activated kinase; MLK: mixed lineage kinase; TAO: thousand and one amino acid protein kinase; ASK: apoptosis signal-regulating kinase; MKK: MAP kinase kinase; Elk: Ets oncogene-related transcription factor 1; RSK: p90 ribosomal S6 kinase; MNK: MAPK-interacting kinase; MSK: mitogen- and stress-activated protein kinase; ATF: activating transcription factor; IRS: insulin receptor substrate; Bcl-2: B-cell leukaemia/lymphoma 2 gene product; MEF: myocyte enhancer factor; MAPKAPK: MAPK-activated protein kinase; CHOP: CCAAT/enhancer-binding protein homologous protein.

and ERK2 is probably the dominant isoform in the lung since the ERK1 knockout phenotype is mild and mostly cognitive in nature compared to wild-type animals. In contrast, ERK2 knockout animals are embryonic lethal early in development. The growth factor signals leading to ERK and JNK bifurcate at the point of Ras activation. However, the signalling pathways are rejoined in the formation of the dimeric transcription factor, activator protein (AP)-1, which comprises the ERK-induced Fos subunits and the JNK-regulated Jun subunits.

Selective inhibitors of ERK have not been described, and most success has been achieved targeting the upstream ERK activator, MAP/ERK kinase (MEK). Furthermore, the application of these compounds has focused on inhibition of abnormal cell proliferation in cancer [16].

The p38 and JNK kinases are often called stress-activated protein kinases because their activity is markedly induced by inflammatory and physical insults. The intracellular signalling pathways leading to p38 and JNK generally bifurcate at the MAP kinase kinase kinase enzymes (fig. 1); however, there is good evidence that MAP kinase kinase (MKK) 4 can also phosphorylate and activate p38 [17]. The p38 and JNK pathways also influence each other at the transcription factor level. For instance, both p38 and JNK can phosphorylate activating transcription factor 2, and p38 activation of myocyte enhancer factor 2C can increase levels of c-Jun [18].

Perhaps the most well-characterised role of p38 is the regulation of tumour necrosis factor (TNF)-α expression *via* increased mRNA stability, a mechanism that may also be augmented by JNK [19, 20]. The regulation of multiple inflammatory cytokines by p38 suggests that p38 inhibitors may be effective in treating some respiratory diseases. In pre-clinical models of lung inflammation, prototype p38 inhibitors, such as SB239063 (trans-1-(4-hydroxycyclohexyl)-4-(fluorophenyl)-5-(2-methoxypyrimidin-4-yl) imidazole), suppressed lung cytokine expression, as well as eosinophil and

TABLE 1 Observations from mitogen-activated protein (MAP) kinase knockout animals

	Phenotype
ERK1	Viable; possible prolonged neuronal synapse
ERK2	Lethal D11; failure of placental development; ERK2 knockdown fibroblasts do not proliferate *in vitro*
ERK5	Lethal D10; endothelial cell failure and vascular leakage in conditional knockout
JNK1	Viable; defect in Th1 differentiation; reduced osteoclastogenesis; sensitivity to induced dermal tumorigenesis; insulin resistance
JNK2	Viable; defect in Th1 differentiation; reduced arthritic joint destruction; resistance to induced dermal tumorigenesis
JNK1+JNK2	Lethal D16; failure in late-stage brain/CNS development; fibroblasts show reduced proliferation *in vitro*; resistant to physical-stress-induced apoptosis; defective TNF-α-induced AP-1 activation
JNK3	Viable; resistant to stress-induced neuronal apoptosis
p38α	Lethal D11; defect in angiogenesis
	Cells resistant to apoptosis induced by Fas; increased transformation in presence of activated Ras; defect in MAPKAPK-2 activation and TNF-α, IL-1 and IL-6 expression
p38β	Viable; no defects observed in the immune responses studied
p38γ	Not reported
p38δ	Not reported

Details of the knockouts are as follows: extracellular signal-regulated kinase (ERK) 1 [1], ERK2 [2], ERK5 [3], c-Jun N-terminal kinase (JNK) 1 [4–6], JNK2 [7–10], JNK1/2 [11], JNK3 [12], p38α [13], and p38β [14]. Th: T-helper cell; CNS: central nervous system; TNF: tumour necrosis factor; AP: activator protein; MAPKAPK: mitogen-activated protein kinase activated protein kinase; IL: interleukin.

neutrophil recruitment [21, 22]. A concerted effort has been made by the pharmaceutical industry to develop p38 inhibitors, and several compounds have progressed into late-stage clinical trials, although not in respiratory disease [23].

C-JUN N-TERMINAL KINASE

Respiratory disease causes broadspread physiological stress in the patient because of widespread cellular stress in the lung. JNK is a stress-activated protein kinase that is activated by environmental insults (*e.g.* osmotic shock, ultraviolet (UV) irradiation, pH changes and reactive oxygen species (ROS)), inflammatory stimuli (*e.g.* antigens, cytokines and infection) and growth factors (*e.g.* vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)) [24]. Since its activation is initiated by such diverse stimuli, JNK probably plays a role in pulmonary diseases of diverse origin, including pollutant-induced bronchitis, allergic and nonallergic asthma, exercise-induced asthma, acute respiratory distress syndrome, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis. Under basal conditions *in vitro* and *in vivo*, it can be experimentally challenging to detect catalytically active JNK. However, the stress stimuli described above can activate JNK tens or hundreds of fold above normal levels. Experimental analysis of JNK activity is typically performed by measuring the phosphorylation state of the target protein, c-Jun. This can be carried out *via* immunoblot analysis of tissue lysates with phospho-specific antibodies directed against c-Jun serine 63/73. Alternative methods include JNK activity assays that measure the rate of phosphorylation of recombinant c-Jun using either radiolabelled adenosine triphosphate (ATP) or the phospho-specific c-Jun antibodies [25].

The JNK1, JNK2 and JNK3 enzymes are highly related and encoded by three separate genes. JNK1 and JNK2 are broadly expressed in tissues, including the lung, whereas JNK3 is expressed exclusively in neurons, cardiac myocytes and testes [12]. Therefore, JNK1 and JNK2 are the relevant isoforms for respiratory disease, although pulmonary hypertension might potentially induce JNK3 activity in the heart [26].

JNKs are intracellular serine-directed protein kinases that phosphorylate, and thereby modulate, the function of target proteins (fig. 2). These target macromolecules include transcription factors, adaptor proteins, cytoskeletal proteins and apoptosis-regulating proteins [27]. JNKs and their upstream kinase activators (MKK4 or MKK7 (level 2) and MAP/ERK kinase kinase (MEKK) or mixed lineage kinase (level 3)) are dynamically complexed by scaffold proteins [28, 29]. It has been shown that specific combinations of JNK/MKK/MEKK–MLK and scaffold protein are probably associated with specific receptor, nucleic-acid-binding, mitochondrial and cytoskeletal proteins such that JNK can integrate diverse environmental stimuli in a selective manner in order to elicit specific cell responses.

JNK was first identified as the enzyme responsible for phosphorylating critical serine residues in the N-terminus of c-Jun, a component of the AP-1 transcription factor [30]. AP-1 is a heterodimer composed of various members of the Jun and Fos families of proteins. By coordinately regulating the expression of potentially hundreds of genes, AP-1 is responsible for the regulation of fundamental physiological processes, such as embryonic development, cell differentiation and transformation, and the acute cellular response to environmental stimuli [27, 31]. Therefore, the regulation of c-Jun/AP-1-dependent gene expression is a central mechanism and was the primary hypothesis regarding how JNK might promote disease. The N-terminal serine residues, serine 63 and 73, of c-Jun are phosphorylation sites uniquely targeted by JNK, and essential for AP-1-directed transcription of genes. Phosphorylation at two other sites, threonine 91 and 93, may also play a role [32]. JNK can phosphorylate other proteins, including, but not limited to, insulin receptor substrate 1 [4], Src-homology collagen protein (Shc) adaptor proteins [33] and

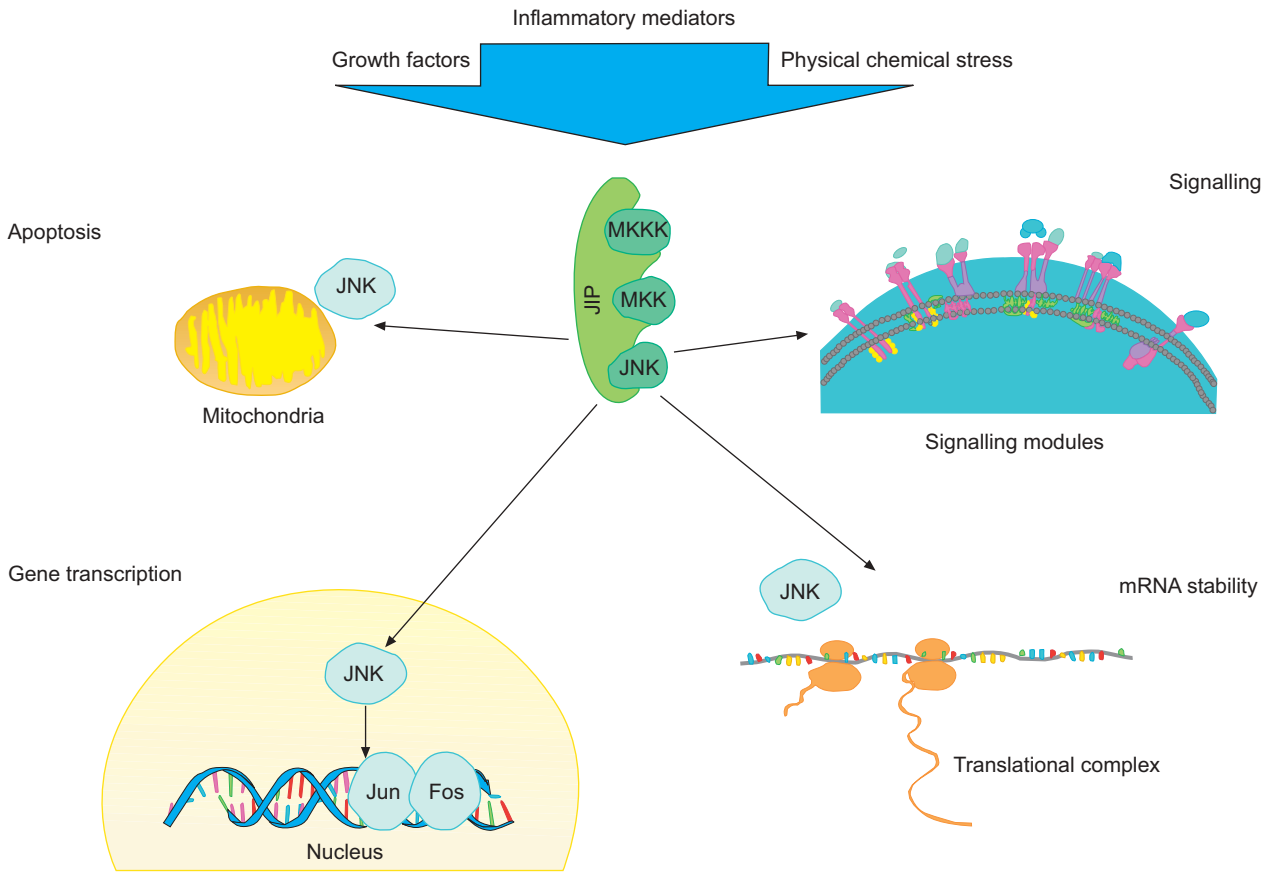

FIGURE 2. c-Jun N-terminal kinase (JNK)-mediated responses in the cell. JNK and its upstream kinase activators are associated with scaffold proteins. Depending on the type, strength and duration of signal, JNK activation may lead to apoptosis *via* B-cell leukaemia/lymphoma gene product (Bcl) family proteins, gene expression *via* transcription factors such as activator protein-1 (Jun/Fos), increased protein expression *via* stabilisation of specific mRNA transcripts and modulated signalling in other pathways, such as *via* insulin receptor substrate 1 in insulin signalling. MKK: mitogen-activated protein kinase kinase; MKKK: MAP kinase kinase kinase; JIP: JNK-interacting protein.

members of the B-cell leukaemia/lymphoma gene product (Bcl) family of apoptosis control proteins [34]. The role of JNK in regulating cell apoptosis is a rapidly expanding field of knowledge, albeit a complicated one, since JNK may exhibit pro- or anti-apoptotic effects, depending on the strength, duration and type of stimulus [35, 36]. Acute injury may cause apoptosis and necrosis with subsequent inflammation, such as in ischaemia-reperfusion injury, or persistent inflammation may result in apoptotic death of organ tissue. As described in more detail below, the role that JNK may play in promoting apoptosis of lung epithelium in diseases such as idiopathic pulmonary fibrosis is intriguing [37]. Much remains to be deciphered regarding how these different, but simultaneous, JNK mechanisms initiate and maintain injury and disease. Genetic models have provided initial insight into JNK-dependent processes, and now unique JNK inhibitors that permit temporal intervention and investigation have been introduced.

INHIBITION OF C-JUN N-TERMINAL KINASE

Low-molecular-weight compounds (<700 Da) represent the most obvious reagents for deriving inhibitors of JNK since small molecules can permeate cell membranes and access intracellular enzymes. Inhibitors exhibiting a modest (10-fold) selectivity for one isoform (JNK2/3) over another (JNK1) have been reported [38], but the discriminatory ability of these compounds has not been demonstrated in cell models. The kinase active site, which is engaged by most of the JNK inhibitors described, is highly homologous across the three isoforms, with perhaps no more than five amino acid differences. The currently available low-molecular weight inhibitors of JNK probably inhibit all JNK isoforms present in the cell. Therefore, although striking phenotypes have been described for *jnk1*$^{-/-}$ or *jnk2*$^{-/-}$ single knockout mice, these have not been fully recapitulated with inhibitors, although inhibitors have shown dramatic efficacy in animal disease models, including cerebral infarction [39] and liver transplantation [40]. A novel *in vivo* chemical genetic approach should permit the pharmacological investigation of isoform-specific inhibition at the experimental level [41].

JNK inhibitors from Celgene (CC-401 (formula undisclosed); Celgene, San Diego, CA, USA) and Serono (AS602801 (formula undisclosed); Serono, Geneva, Switzerland) are currently undergoing phase 1 clinical trials and thus more may soon be learnt about the activity of JNK inhibitors in human disease. Alternative therapeutic strategies may also be possible, particularly for respiratory disease, since inhalation provides targeted tissue delivery. These strategies include viral or chemical transfer of antisense oligonucleotide decoys and

small interfering RNA. Another intriguing alternative is the use of a JNK decoy peptide that has demonstrated vivid efficacy in a number of cell and animal models [39, 42]. An updated description of JNK inhibitors is provided in [43].

In 2001, SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) was reported as the first low-molecular-weight inhibitor of JNK to show selectivity *versus* the related MAP kinase members, p38 and ERK [20]. Used in concert with available MEK (ERK-activating kinase) and p38 inhibitors, it has been a powerful pharmacological tool for discriminating the role of these respective pathways. However, as is not uncommon for ATP-competitive inhibitors, it is not an entirely selective protein kinase inhibitor [44]. Therefore, conclusions obtained using this compound should be in the context of the study and in association with observations made using other methods of inhibition where possible.

SP600125 activity has been reported in a number of rodent models of pulmonary disease, including single and chronic allergen challenge in the rat [45, 46] and mouse [47], and bleomycin-induced injury and repair in the mouse [48]. Some of these studies have been repeated and confirmed using proprietary JNK inhibitors from a distinct chemical class, a JNK inhibitor from a discovery programme at Serono [49, 50] and AP-1 activation inhibitors [51]. These studies revealed both expected and unexpected positive treatment effects. Based on the biology of AP-1 and the role of JNK in T-cell differentiation, it was assumed that JNK inhibitors might reduce the leukocyte infiltrate as well as the expression of cytokines and chemokines. Less expected were the effects on airway smooth muscle goblet cells and deposition of extracellular matrix. These latter observations have expanded the potential role of JNK inhibitors in pulmonary disease.

Defining which inflammatory enzymes, cytokines or modulators are JNK-dependent is fraught with complexity because it is clear that stimulus, cell type and other regulatory signalling enzymes all affect the final outcome of protein expression. Furthermore, if JNK affects the transformation of a cell type, then it will, by default, affect the expression of proteins that are only expressed in the differentiated phenotype. Therefore, this review focuses on the role of JNK in several general mechanisms that are all key to the progression of respiratory disease: inflammation, proliferation, differentiation, and apoptosis. Where possible, specific examples of regulated genes and proteins in lung tissue or cell types associated with lung pathologies are highlighted.

INFLAMMATION

Inflammation is the primary initiating pathology in many lung diseases. Owing to the central role of AP-1 in regulating cytokine and inflammatory gene expression, it is highly likely that JNK is important in the genesis of many pro-inflammatory mediators. In a mouse model of chronic lung inflammation (allergic inflammation), significant inhibition of TNF-α, interleukin (IL)-4, IL-13 and RANTES (regulated on activation, normal T-cell expressed and secreted) in lung homogenates was observed with JNK inhibitor SP600125 [47]. Cytokines and chemokines are believed to play a major role in T-helper cell (Th) type-2-mediated respiratory disease and exhibit elevated levels in bronchial biopsy specimens and sputum from patients. In a single allergen challenge model, inhibition of a large number of cytokine mRNAs in lung tissue was observed [45]. This is consistent with the described effects of SP600125 in isolated leukocytes [20]. JNK-dependent cytokine expression may also be regulated by mRNA stabilisation, which acts as an auxiliary mechanism of increasing the amount of expressed and secreted cytokine. JNK-dependent stabilisation of mRNA has been shown for IL-2, IL-3, TNF-α and cyclooxygenase 2 and it is likely that additional stabilised mRNA species will be identified [52, 53].

Targeted deletion of *jnk1* and *jnk2* has confirmed a role for JNK in regulating the immune response. Both *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice showed a bias towards formation of Th2, although the proposed mechanisms were distinct. In *jnk1*$^{-/-}$ cells, phosphorylation of nuclear factor of activated T-cells 2 (NF-AT2) was markedly reduced, resulting in elevated levels of nuclear NF-AT2 and enhanced IL-4 expression and Th2 differentiation [5]. In *jnk2*$^{-/-}$ cells, it was proposed that a lack of IL-12 receptor subunit β2 prevents IL-12-mediated Th1 development, resulting in default to Th2 [7]. It was also apparent, from these studies, that deficiency in either JNK isoform resulted in abnormal cytokine expression. Generation of JNK-deficient peripheral T-cells in adult mice using rag deletion methodology showed increased expression of Th2-type cytokines, such as IL-4, -5 and -13, in CD3/CD28-stimulated cells; however, it was noted that peripheral lymphocyte populations appeared normal *in vivo* [54]. From these data, it might be concluded that pharmacological inhibition of JNK should lead directly to enhanced Th2 accumulation and potential exacerbation of allergic pathology and asthma. However, in the rodent models of lung inflammation, consistent inhibition of Th2 cytokine expression and the lymphocytic infiltrate in the airways was observed. This suggests that suppression of JNK activity is not equivalent to complete removal of JNK (knockout), inhibition of both JNK isoforms in T-cells is different to inhibition of either isoform alone, or JNK inhibition of other cell types exerts secondary effects on T-cells [55]. Clearly, the use of genetically modified animals in a disease setting with JNK inhibitors will be helpful.

Although T-cells are critical for regulating the immune response, in many situations it is the resident macrophage or dendritic cell that first detects antigen and initiates this response. The airway represents an exposed epithelial surface of far greater total area than the skin and is therefore subject to diverse infectious pathogens and allergens. In normal human subjects, macrophages comprise 90% of all airway leukocytes and the absolute number of macrophages increases in respiratory disease, such as asthma and COPD. However, *in vivo*, JNK inhibitors exerted only a modest effect on airway macrophage numbers compared to the significant inhibition of eosinophils and T-cells. JNK inhibitors may exert their strongest effect on macrophages by blocking the expression of cytokines such as TNF-α [20, 56].

C-JUN N-TERMINAL KINASE INHIBITORS AND GLUCOCORTICOIDS

Corticosteroid treatment represents the gold-standard anti-inflammatory therapy for many pulmonary diseases, including asthma. Both JNK inhibitors and steroids, such as dexamethasone, inhibit many of the same pro-inflammatory genes,

including TNF-α, IL-4, IL-13, monocyte chemoattractant protein-1 and RANTES. This commonality may not be a coincidence but instead rooted in mechanism. There are a striking number of reported interactions between glucocorticoid receptor (GR) activity and the JNK pathway. First, GR may bind directly to the active AP-1 transcriptional complex and suppress expression of these genes (transrepression). Secondly, the activity of GR as a positive regulator of genes (transactivation), inducing those such as the phosphatase MAP kinase phosphatase-1, may lead to feedback which dephosphorylates and inactivates JNK [57, 58]. Thirdly, GR monomer has been shown to bind cytoplasmic JNK and prevent association of JNK with its upstream activators, MKK4 and MKK7 [59]. Furthermore, GR-ligated JNK can translocate to the nucleus *via* GR chaperones, resulting in inactive JNK in the nucleus that may compete with active JNK for binding to c-Jun. Consistent with these mechanistic proposals is the observation that corticosteroid-resistant asthmatics exhibit increased levels of JNK signalling pathway proteins and heightened JNK activity [60]. A similar observation has been made in patients with Crohn's disease [61]. Whether or not JNK inhibitors represent an alternative therapy for this difficult-to-treat group of patients remains to be seen.

PROLIFERATION

A novel observation in chronically allergen-challenged rats and mice was that JNK inhibitor caused significant inhibition of both airway smooth muscle proliferation and goblet cell hyperplasia, which are common features of the asthmatic airway [46, 47]. It remains unclear whether this effect of JNK inhibitor was due to inhibition of growth factor expression or *via* direct inhibition of cell cycle mechanisms. However, decreasing the thickness of the smooth muscle layer may contribute to the decrease in airway hyperresponsiveness and reduction in goblet cells to give decreased mucus production observed in these studies.

Airway smooth muscle cells contain a responsive JNK signalling pathway, as shown by the increased phosphorylation of JNK and c-Jun following TNF-α, IL-1β or IL-4/13 stimulation, and the inhibition of RANTES and granulocyte-macrophage colony-stimulating factor (GM-CSF) expression after treatment with JNK inhibitor [62, 63]. SP600125 has also been shown to inhibit the proliferation of aortic vascular smooth muscle cells [64].

In vitro data suggest that p38 and ERK may also play important independent roles in airway smooth muscle cell proliferation, as demonstrated with selective low-molecular-weight inhibitors of their respective pathways [65]. A role for JNK in proliferation and cell transformation has been suspected for a long time. Soon after its discovery, and before the identification of JNK, c-Jun was described as a proto-oncogene [66]. Support for the importance of the JNK signalling pathway in regulating aspects of cell growth has come from the negative and positive regulation of skin tumours in *jnk1*$^{-/-}$ and *jnk2*$^{-/-}$ mice respectively [8, 67], reduced proliferation of *jnk1*$^{-/-}$ *jnk2*$^{-/-}$ double-knockout fibroblasts [11], and involvement of JNK in Ras- and p53-mediated regulation of cell proliferation [68, 69].

However, how essential JNK is in regulating cell growth in nontransformed cell types remains undefined. JNK appears to be critical in liver regeneration [70, 71], fibroblast proliferation [11] and erythroid progenitor maturation [72], but not in tubular epithelial cells of the kidney [73]. Recent data also suggest that, at least in fibroblasts, JNK1 and JNK2 may play unique roles with respect to proliferation. Although JNK1 is induced upon stress and may promote c-Jun-dependent proliferation, JNK2 binds Jun under basal conditions and promotes its degradation. These data suggest JNK2-mediated negative regulation of Jun [74]. Therefore, within any one organ, the proliferation of distinct cell types that comprise a tissue may be differentially modulated by JNK and affected by JNK inhibitors. With respect to therapeutic intervention in the lung, it is important to preserve the ability of epithelium to regenerate while suppressing fibroblast and smooth muscle proliferation. Whether both sides of this ideal outcome are possible awaits further evaluation.

MYOFIBROBLAST ACTIVITY

Regeneration and fibroplasia are two essential mechanisms for tissue remodelling and repair. In regeneration, the damaged or lost tissue is replaced by tissue of the same type. In fibrosis, abnormal tissue is formed by the proliferation of fibroblasts, deposition of matrix proteins and formation of excess connective tissue. Fibrosis, and in particular exacerbated fibrosis, is a critical pathology of several lung diseases. *In vivo* data demonstrating antifibrotic activity through suppression of JNK are scant, although pre-clinical studies are ongoing. It has been reported that IL-4 and IL-13 act through the JNK pathway to activate human lung fibroblasts [75]. Two distinct low-molecular weight inhibitors of JNK showed equivalent inhibition of fibrosis in the bleomycin-induced lung injury model in two independent studies [48, 50]. Adenoviral delivery of a JNK dominant-negative mutant into mouse alveolar epithelial cells *in vitro* prevented bleomycin-induced apoptosis and activation of mitochondrial Bcl-2-associated X protein [76]. Striking data have also been obtained showing inhibition of renal fibrosis by CC-401, a JNK inhibitor that has reached clinical trials [40, 77]. This early work extends the potential of JNK inhibitors from asthma into other pathologies, such as COPD and idiopathic pulmonary fibrosis.

Fibrosis is driven by the activity of myofibroblasts. Resident fibroblasts are a common cell type in many tissues, but are normally present as mostly quiescent cells maintaining tissue rigidity and architecture. In contrast, myofibroblasts show an altered phenotype, including the expression of muscle proteins such as α-actin, and secretion of large amounts of matrix proteins, such as collagens, fibronectin and elastin. Transforming growth factor (TGF)-β is a master molecule of fibroblast activity and wound healing. TGF-β ligation to specific cell surface receptors leads to phosphorylation, dimerisation and nuclear translocation of a unique class of transcription factors called Smads (mothers against decapentaplegic homologues (*Drosophila*)), which bind DNA and interact with multiple other DNA-binding proteins, including c-Jun/AP-1. TGF-β is also a potent activator of JNK, and preliminary evidence suggests that JNK may regulate the expression of connective tissue growth factor (CTGF), a key secondary effector of TGF-β [65, 77]. There are at least three possible mechanisms whereby JNK might regulate myofibroblast activity. The first is the chemotactic recruitment of

myofibroblasts to the site of injury. JNK may play a role in promoting the expression and function of chemotactic mediators such as PDGF, epidermal growth factor (EGF) and TNF-α, and may also be essential for fibroblast motility [78]. The second is the differentiation process of fibroblast to myofibroblast, since, again, PDGF, EGF and TNF-α help to drive this process. The third mechanism is the transcriptional regulation of fibrotic genes containing AP-1 regulatory elements, including fibronectin, VEGF and CTGF [79, 80]. JNK is a negative regulator of TGF-β derived from embryonic fibroblasts, implying that JNK does not induce fibrosis by increasing production of autocrine TGF-β. However, in these cells, JNK showed differential effects on other fibrotic genes. Procollagen VI, fibronectin and VEGF mRNA levels were decreased in *jnk*$^{-/-}$ fibroblasts, consistent with previous reports that these genes are positively regulated by AP-1 [81]. Furthermore, the JNK inhibitor SP600125 blocked TGF-β expression in human lung epithelial cells [82], suggesting different regulation in embryonic compared to adult cells and/or fibroblasts compared to epithelial cells.

It has been reported that the initiation of pulmonary fibrosis by TGF-β is associated with apoptosis of the epithelium, and that inhibition of epithelial apoptosis directly suppresses the fibrosis and remodelling. Interestingly, a null mutation in the early stress response gene, early growth response (Egr)-1, has been used to inhibit apoptosis [37]. In lung tissue from COPD patients, Egr-1, along with CTGF and TGF-β, was identified as one of a group of significantly upregulated genes compared to non-COPD controls [83]. It is provocative to propose that JNK may be intricately involved in this process because Egr-1 is a JNK/AP-1-regulated gene [84].

REGULATION OF APOPTOSIS

Apoptotic cell death is a fundamental process necessary for tissue modelling, immune cell maturation and selection, and normal cell turnover. Following severe stress, excessive apoptosis can lead to organ dysfunction and death. Early on, JNK was identified as a UV-induced kinase [30], and it was later shown that *jnk1*$^{-/-}$ *jnk2*$^{-/-}$ fibroblasts were resistant to UV-induced cell death, indicating that JNK is necessary for apoptosis following radiation [11]. The role of JNK in promoting apoptosis is dependent upon the source and duration of stimulus, cell type and interplay with anti-apoptotic pathways, particularly those mediated by nuclear factor (NF)-κB [35]. Although the precise mechanisms require full elucidation, it is likely that JNK promotes cell death by activating apoptosis-enhancing mitochondrial Bcl proteins such as BH3-interacting domain death agonist (Bid) and Bcl-2-like 11 (Bim), and suppressing the function of anti-apoptotic members such as Bcl-2 [85]. The resulting permeabilisation of the outer mitochondrial membrane leads to a cascade of molecular events involving the release of cytochrome c into the cytoplasm, formation of the apoptotic protease activating factor (Apaf) 1 apoptosome, activation of caspase 9 and consequent cleavage of the executioner procaspases 3, 6 and 7. Additional mitochondrial apoptogenic proteins, such as second mitochondrial activator of apoptosis (Smac) and direct inhibitor-of-apoptosis-binding protein with low pI (Diablo), act to inhibit the action of NF-κB-regulated inhibitors of apoptosis [86].

Therefore, JNK-mediated pro-apoptotic activity is a potent enhancer of the classic caspase death pathway. Evidence for these JNK-associated mechanisms has been collected in cigarette-smoke-induced lung apoptosis in rats [87]. Tissue damage caused by ROS is an important injury mechanism in lung injury induced by pollutants (*e.g.* cigarette smoke) and as a by-product of the activated leukocyte infiltrate [88]. The generation of ROS is also a hallmark of ischaemia-reperfusion injury, which may occur unexpectedly (*e.g.* pulmonary embolism) or as a consequence of surgery (*e.g.* lung transplantation). It has been shown that, although the ischaemic time predetermines the extent of injury, JNK is induced rapidly upon the reperfusion event, primarily due to the sudden osmotic stress that occurs in the local environment around the cell, as well as the rapid reoxygenation and consequent generation of ROS [89]. JNK and AP-1 are activated in transplanted liver and lung, and JNK inhibitors minimise tissue damage and enhance survival in these models [40, 90]. Inhibition of JNK using a stably expressed dominant-negative mutant of JNK effectively prevented apoptosis of lung epithelial cells exposed to a hyperoxic (95% oxygen) environment [91].

ROLE OF C-JUN N-TERMINAL KINASE IN OTHER INFLAMMATORY CELL TYPES

The role of JNK in certain specific cell types central to respiratory disease has not yet been covered in the present review because, in general, they are poorly understood. Eosinophils are central to allergic disease, and it has been noted that cytokines such as GM-CSF, IL-5 and TNF-α act on eosinophils to prolong their survival [92]. Withdrawal of IL-5 leads to apoptosis, but it is not known whether this event requires JNK, although JNK is involved in eosinophil apoptosis due to oxidant insult [93, 94]. *In vivo* observations indicate that inhibition of JNK can suppress eosinophilic infiltration into tissue and airways [45, 46]. Ligation of the high-affinity immunoglobulin E receptor by immunoglobulin E is a potent stimulator of JNK in mast cells and basophils [95, 96]; however, it is not really known what effect JNK activation has on the release of mast cell mediators that trigger the allergic and asthmatic response. JNK plays a role in mast cell IL-3 mRNA stabilisation and is potentially involved in mast cell proliferation, but probably not in histamine release [53]. Neutrophils express many early response genes that promote the inflammatory response. Low-molecular-weight JNK inhibitors were less effective than expected in animal models exhibiting prominent neutrophila. These models included the rat air pouch model, carrageenan-induced paw oedema and endotoxin-induced pulmonary neutrophilia (data not shown). It has been reported that, although neutrophils contain JNK, stimuli such as lipopolysaccharide, TNF-α and phorbol myristate acetate, which activate JNK in other cell types, do not activate JNK in neutrophils, and SP600125 did not inhibit the expression of these cytokines in neutrophils [97]. Similarly, no effect on *N*-formyl-methionyl-leucyl-phenylalanine-induced leukotriene release from neutrophils was observed by the present author using a range of JNK inhibitors (data not shown). Therefore, the coupling of immune receptors to the JNK pathway appears unique in neutrophils compared to other leukocytes.

CONCLUSIONS

JNK was first characterised as a stress-activated protein kinase in the early 1990s. Although its potential as a drug target was immediately appreciated, the utility of JNK inhibitors has evolved with time and experience. Initially, it was proposed that inhibition of AP-1 would provide the dominant therapeutic benefit *via* broad anti-inflammatory activity. The subsequent generation of JNK knockout animals highlighted a role for JNK in promoting apoptosis under select conditions. Still later, the evaluation of novel pharmacological agents has revealed a role for JNK in fibroblast differentiation and function. Each of these pathological mechanisms is evident in diseases of the lung.

Respiratory diseases represent a growing proportion of the total health burden, suggesting either that susceptibility is increasing and/or current medications are not effectively treating all new cases. Not surprisingly, recently approved therapies (improved glucocorticoids, leukotriene antagonists, immunoglobulin E and, perhaps soon, phosphodiesterase 4 inhibitors) have been rapidly adopted. These new drugs also illustrate the variety of disease-promoting molecules that can be targeted to provide an effective therapy. The stress-activated protein kinase, c-Jun N-terminal kinase, is a relevant target for the next generation of drug candidates because of its pluripotent mechanisms that are manifest in a variety of pathologies seen in respiratory diseases.

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