**c-Jun N-terminal kinase mediates constitutive human eosinophil apoptosis**

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**Abstract**

Eosinophils are considered to play an important role in the pathogenesis of asthma. Glucocorticoids are potent anti-inflammatory agents for the treatment of chronic inflammatory diseases and they have been shown to increase the rate of eosinophil apoptosis. c-Jun N-terminal kinase (JNK) has been suggested to participate in the signaling pathways of apoptosis. The aims of the present study were to examine whether JNK is involved in the regulation of constitutive eosinophil apoptosis and whether it mediates dexamethasone-induced apoptosis of human eosinophils.

Isolated human eosinophils were cultured with and without dexamethasone and the JNK inhibitor L-JNKI-1. Apoptosis was assessed by measuring the relative DNA content of propidium iodide-stained cells and confirmed by Annexin V-binding and morphological analysis with bright field microscopy. The phosphorylation of both JNK and c-Jun were measured by Western blotting.

During a 40 h culture, dexamethasone (1 \( \text{mM} \)) enhanced human eosinophil apoptosis by 10–30%. Culture with L-JNKI1 (10 \( \text{mM} \)) inhibited apoptosis in dexamethasone-treated cells by 53%. Furthermore, L-JNKI1 decreased the rate of constitutive eosinophil apoptosis by 64%. However, the enhancement of eosinophil apoptosis by dexamethasone was not reversed by L-JNKI1. Slow activation of JNK in constitutive apoptosis as well as a similar tendency in dexamethasone-induced eosinophil apoptosis could be observed by Western blot analyses. c-Jun was found to be active both in the presence and absence of dexamethasone. However, no further phosphorylation of the serine residue 63 of c-Jun could be seen.

Taken together, our present results suggest that JNK is active during apoptosis of human eosinophils both in the presence and absence of glucocorticoids. JNK seems to mediate constitutive human eosinophil apoptosis. However, the activity of JNK is not enhanced by glucocorticoids and the effects of glucocorticoids cannot be reversed by JNK inhibition. JNK therefore seems not to mediate glucocorticoid-induced human eosinophil apoptosis.

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**Keywords:** Eosinophils; Apoptosis; JNK; c-Jun; Glucocorticoids

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**1. Introduction**

Eosinophils have been implicated in the pathogenesis of allergic inflammatory diseases such as bronchial asthma and allergic rhinitis \([1,2]\). Activation and degranulation of eosinophils in the airways is considered to cause epithelial tissue injury and airway remodelling \([3–5]\). Eosinophil apoptosis or programmed cell death has been suggested as a major mechanism in the resolution of eosinophilic inflammation for example in asthma \([6–8]\). Moreover, it
has been demonstrated that eosinophil apoptosis is delayed in asthmatic patients [9]. Apoptosis is characterized by specific biochemical and morphological changes such as cell shrinkage, nuclear coalescence, chromatin condensation and endonuclease-catalyzed DNA breakdown followed by fragmentation of the cell into discrete apoptotic bodies, which are phagocytosed intact without releasing their granule contents to the surrounding tissues, which might induce inflammation [7,10,11]. In the absence of cytokines, in vitro cultured human eosinophils undergo apoptosis that can be inhibited by different cytokines, principally interleukin (IL-) 3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) [1,8,11,12].

Glucocorticoids are commonly used anti-inflammatory agents for the treatment of asthma and several other chronic inflammatory diseases [13]. Glucocorticoids decrease the transcription of many inflammatory cytokines, such as IL-5, tumour necrosis factor (TNF-α) and GM-CSF, and reduce the numbers and activation of airway inflammatory cells [14]. The target cells include eosinophils, neutrophils, T-lymphocytes, macrophages, mast cells, dendritic cells, epithelial cells and endothelial cells [14,15]. Inhaled glucocorticoids have been reported to reduce the numbers of tissue and blood eosinophils [15]. Consistently, clinically relevant concentrations of glucocorticoids have been shown to enhance eosinophil apoptosis in vitro [16,17]. The induction of eosinophil apoptosis may therefore constitute one of the anti-inflammatory actions of glucocorticoids in allergy and asthma. The mechanisms by which glucocorticoids promote eosinophil apoptosis are currently, however, poorly understood.

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that are characteristically activated by threonine and tyrosine phosphorylation. In mammals, three major MAPK subfamilies have been identified, the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 MAP kinase (p38 MAPK) [18]. Both p38 MAPK and JNK have been proposed to play a role in the signaling pathways of apoptosis [18]. It has previously been shown that p38 MAPK activity is involved in the inhibition of human eosinophil apoptosis [19]. JNK is activated by dual phosphorylation induced by cytokines or environmental stress (e.g. oxidative stress) [18,20]. The signaling cascades that lead to the regulation of apoptosis after JNK activation are not fully understood but a critical role for JNK has been suggested in the regulation of activator protein (AP) -1 transcriptional activity, and phosphorylation of c-Jun on serine 63 and 73 residues by JNK has been found to increase its transcriptional activity [18]. Recent evidence suggests that nitric oxide and glucocorticoids may activate MAP kinases in human eosinophils [21–23]. Activation of JNK by dexamethasone was associated with induction of eosinophil apoptosis, a process that could be inhibited by GM-CSF and by blocking JNK activation by a JNK inhibitor (SP600125) [21]. However, the results concerning the importance of JNK activation in glucocorticoid-induced eosinophil apoptosis remain controversial as JNK1/2 antisense phosphorothioate oligodeoxynucleotides did not exert any significant effect on dexamethasone-induced eosinophil apoptosis [22].

Given the possible role of JNK in regulating apoptosis of eosinophils and other cell types, the aim of the present study was to elucidate the role of JNK in spontaneous and dexamethasone-enhanced apoptosis of human eosinophils.

2. Materials and methods

2.1. Eosinophil isolation

One hundred millilitres of peripheral venous blood was obtained from healthy or atopic volunteers. Before donating blood, the volunteers gave written informed consent to a study protocol approved by the Ethics committee of Tampere University Hospital (Tampere, Finland). Eosinophils were isolated under sterile conditions to >99% purity as previously described [9,19,23,24]. Eosinophils were cultured (+37°C, 5% CO2) for 40 h in the absence and presence of dexamethasone and a JNK inhibitor in RPMI 1640 medium (Dutch modification) with 10% fetal calf serum and antibiotics.

2.2. Determination of apoptosis by the relative DNA content assay

The percentage of apoptotic cells was assessed by measuring the relative DNA content by flow cytometry (FACScan, Becton Dickinson, San Hose, CA) of propidium iodide (PI)-stained cells as previously described [9,17,24]. Endonuclease-catalysed DNA fragmentation is considered to be a specific feature of apoptosis [24]. Therefore, the cells showing decreased relative DNA content were considered as apoptotic. Eosinophils were suspended in 300 µl of hypotonic propidium iodide solution (25 µg ml⁻¹ in 0.1% sodium citrate and 0.1% Triton X-100), protected from light and incubated at +4°C for 1 h before flow cytometric analysis.

2.3. Annexin V-FITC

Annexin-V binding and analysis by flow cytometry were performed as previously reported [17]. Briefly, after 18 h incubation, the cells were washed in PBS solution and suspended in binding solution (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). The mixture of 5 µl annexin V-FITC (solution containing 50 mM Tris, 100 mM NaCl, 1% BSA, sodium azide, pH 7.4) and 195 µl of the 5 x 10⁶ cells/ml cell suspension was incubated at room temperature for 10 min. The cells were washed and resuspended in 190 µl of binding buffer, and 10 µl of 20 µg ml⁻¹ propidium iodide solution was added. Annexin-positive cells were considered to be apoptotic.
2.4. Morphological analysis

For morphological analysis, eosinophils were spun onto cytospin slides (500 rev min$^{-1}$, 5 min) and stained with May–Grünwald–Giemsa after fixation in methanol. Cells showing the typical features of apoptosis, such as cell shrinkage, nuclear coalescence and chromatin condensation, were considered as apoptotic [24].

2.5. Western blotting

Eosinophils were suspended at 10$^6$ cells/ml and cultured at +37°C. At indicated time points, the samples were centrifuged at 12,000g for 15 s. The cell pellet was lysed by boiling for 5 min in 30 μl of Laemmli sample buffer. The sample was centrifuged at 12,000g for 10 min and the debris was carefully removed. Samples were stored at −20°C until the Western blot analysis. The protein sample (30 μl) was loaded onto 10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 2 h at 100 V. The separated proteins were transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) with a semidry blotter at 2.5 mA cm$^{-2}$ for 60 min. After transfer, the membranes were blocked by 5% bovine serum albumin (BSA) in TBST (20 mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature and incubated with the specific primary antibody overnight at +4°C in the blocking solution. The membrane was thereafter washed 4× with TBST for 5 min, incubated for 30 min at room temperature with the secondary antibody in the blocking solution and washed 4× with TBST for 5 min. Bound antibody was detected by using SuperSignal West Dura chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The chemiluminescent signal was quantified by using the FluorChem software version 3.1.

2.6. Materials

L-JNKI1 (c-Jun N-terminal kinase peptide inhibitor 1, L-stereoisomer) and L-TAT control peptide were purchased from Alexis Corp. (Läufelfingen, Switzerland). c-Jun and JNK1 antibodies and goat anti-rabbit polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). phospho-c-Jun (Ser63) antibody was from Cell Signaling Technology, Inc. (Beverly, MA, USA) and phospho-JNK (Thr183/Tyr185, Thr221/Tyr223) antibody was from Upstate (Lake Placid, NY, USA). Other reagents were obtained as follows: propidium iodide (Tocris, Bristol, UK), anti-CD16 microbeads and the magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany), Ficoll-Paque (Pharmacia AB, Upsala, Sweden), antibiotics and RPMI 1640 (Dutch modification) (Gibco BRL, Paisley, UK), fetal calf serum, Hank’s balanced salt solution (HBSS) and RPMI 1640 (BioWhittaker, Verviers, Belgium), Annexin V-FITC kit (Bender medSystems, Vienna, Austria), May–Grünwald (Merck, Darmstadt, Germany) and Giemsa (J.T.Baker, Deventer, Holland). Dexamethasone and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Dexamethasone, L-JNKI1 and L-TAT were dissolved in HBSS.

2.7. Statistics

All results are expressed as the mean±SEM. Apoptosis is expressed as an apoptotic index (number of apoptotic cells/total number of cells, i.e. apoptotic index 0.1 means 10% of the cells are apoptotic). Statistical significance was calculated by analysis of variance for repeated measures supported by the Dunnett test or by paired t-tests. Differences were regarded as significant when $p<0.05$.

3. Results

3.1. Effect of JNK-inhibition on constitutive eosinophil apoptosis

Human eosinophils cultured for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis. The constitutive apoptotic index was 0.36±0.07 ($n=6$) as defined by the relative DNA fragmentation assay in propidium iodide-staining. The novel cell-permeable JNK inhibitor peptide L-JNKI1 [25,26] (10 μM) was found to decrease constitutive eosinophil apoptosis by 64% when apoptosis was assessed by using the relative DNA fragmentation assay (Fig. 1). The negative control peptide L-TAT did not affect apoptosis as compared with medium control ($n=6$, data not shown). The flow cytometry histograms of eosinophils treated with L-JNKI1 clearly demonstrate the reduction in the numbers of cells with decreased relative DNA content i.e. reduced apoptosis by L-JNKI1 as compared with the negative control L-TAT (Fig. 2A and B). When apoptosis was assessed by measuring phosphatidyserine expression (i.e. Annexin V-binding), the effect was similar as L-JNKI1 decreased apoptosis by 40% (Fig. 2C and D). The apoptotic indices were 0.25±0.02 and 0.15±0.01 ($p<0.001$, $n=8$) for L-TAT and L-JNKI1, respectively. Consistently, L-JNKI1 also reduced the number of eosinophils showing the typical characteristics of apoptosis such as cell shrinkage, chromatin condensation and nuclear coalescence. The apoptotic indices were 0.61±0.07 and 0.46±0.05 in the presence of L-TAT and L-JNKI1, respectively ($p<0.01$, $n=6$, Fig. 2E and F).

3.2. Effect of JNK-inhibition on dexamethasone-enhanced eosinophil apoptosis

Human eosinophils cultured for 40 h in cytokine-deprived conditions underwent spontaneous apoptosis (apoptotic index 0.36±0.07). In the presence of
Dexamethasone (1 μM), the apoptotic index was enhanced up to 0.43 ± 0.06 (n = 6) when analyzed by using the relative DNA fragmentation assay. L-JNKI1 (10 μM), but not the negative control peptide L-TAT, was found to decrease dexamethasone-induced eosinophil apoptosis by 53% (Fig. 3). The flow cytometry histograms of dexamethasone-enhanced eosinophil apoptosis in the presence of L-JNKI1 show the decrease in the number of cells showing hypodiploid DNA content suggesting reduced apoptosis by L-JNKI1 as compared with L-TAT (Fig. 2G and H). When Annexin V-binding was used as an indicator of apoptosis, the results were similar as L-JNKI1 decreased eosinophil apoptosis in the presence of dexamethasone by 37% (Fig. 2I and J). The apoptotic indices were 0.29 ± 0.02 and 0.18 ± 0.02 (p < 0.001, n = 8) in the presence of L-TAT and L-JNKI1, respectively. Consistently, L-JNKI1 reduced eosinophil apoptosis in the presence of dexamethasone as assessed by morphological analysis by bright field microscopy. The apoptotic indices were 0.74 ± 0.06 and 0.65 ± 0.05 with L-TAT and L-JNKI1, respectively (p < 0.05, n = 6, Fig. 2K and L).

3.3. Fold-increase in eosinophil apoptosis by dexamethasone

The JNK inhibitor L-JNKI1 delays human eosinophil apoptosis both in the presence and absence of dexamethasone by 53% and 64%, respectively. As the JNK inhibitor seems to reduce apoptosis to a similar extent in the presence and absence of dexamethasone, we calculated how many fold does dexamethasone increase apoptosis in the absence and presence of the JNK inhibitor. These fold-increased rates of eosinophil apoptosis (apoptotic index in the presence of dexamethasone/apoptotic index in the absence of dexamethasone) showed that L-JNKI1 did not reverse the steroid-effect (Fig. 4), suggesting that the enhancement of eosinophil apoptosis by dexamethasone may not be mediated through activation of JNK.

3.4. JNK activation during constitutive and dexamethasone-enhanced eosinophil apoptosis

Dexamethasone has been proposed to activate JNK in human eosinophils [21,22]. To further evaluate the possible role of JNK in spontaneous and dexamethasone-induced eosinophil apoptosis, the activation of JNK was studied. The activity of JNK was assessed by calculating the ratio of phosphorylated/total JNK by using antibodies directed against Thr183/Tyr185 and Thr221/Tyr223 phosphorylated (activated) JNK and total JNK, which recognize JNK at molecular weights of 46 and 55 kDa. In freshly isolated human eosinophils, a spontaneously active JNK could be seen (Fig. 5A). During spontaneously occurring eosinophil apoptosis, a slow activation of 55 kDa JNK could be observed (Fig. 5A). In the presence of dexamethasone, a non-significant tendency towards slow activation of JNK could be seen (Fig. 5A). This suggests that the JNK is slowly activated during human eosinophil apoptosis but steroids do not increase JNK activity. In addition, an analysis of a downstream effector of JNK was conducted. c-Jun is an inducible transcription factor and can be activated by phosphorylation at specific serine 63 and 73 residues only by JNK [27]. The activity of c-Jun was analyzed by Western blotting by using antibodies directed against Ser-63 phosphorylated c-Jun and total c-Jun. Similarly to the active JNK seen in freshly isolated human eosinophils, a phosphorylated c-Jun could be detected in these cells. However, during constitutive apoptosis of eosinophils, c-Jun phosphorylation at the serine 63 residue was not increased (Fig. 6A). Dexamethasone (1 μM) did not increase c-Jun phosphorylation (Fig. 6B), which further suggests that dexamethasone does not induce further activation of JNK.

4. Discussion

We have shown here that JNK mediates spontaneous eosinophil apoptosis and that inhibition of the JNK pathway leads to increased survival of human eosinophils. The JNK inhibitor L-JNKI1 markedly reduced the rate of human eosinophil apoptosis. These effects could be observed with each method used to measure apoptosis, the relative DNA fragmentation assay, Annexin V-binding and morphological analysis by bright field microscopy. Moreover, the presence of active JNK as evidenced by the presence of phosphorylated JNK as well as phosphorylated...
c-Jun in freshly isolated cells and the slight activation of JNK during spontaneously occurring apoptosis support the hypothesis that JNK mediates constitutive apoptosis of human eosinophils. Furthermore, in line with our results, Gardai and co-workers have reported that the JNK inhibitor SP600125 prevents dexamethasone-induced...
eosinophil apoptosis while a similar although non-significant anti-apoptotic tendency could be observed in the absence of dexamethasone \((n = 3)\) [21].

In addition to decreasing constitutive apoptosis, inhibition of the JNK pathway was in the present study found to diminish eosinophil apoptosis in the presence of dexamethasone. By calculating the ratios of how many fold does dexamethasone increase apoptosis in the absence and presence of the JNK inhibitor L-JNKI1, we concluded that JNK inhibition did not, however, reverse the steroid effect indicating that the JNK may not mediate glucocorticoid-induced apoptosis in human eosinophils as recently suggested by Gardai et al. [21]. In conjunction with our present results, Zhang et al. [22] reported that inhibition of JNK by JNK1/2 antisense oligodeoxynucleotides did not exert any significant effects on dexamethasone-induced eosinophil apoptosis. Dexamethasone has previously been shown to activate JNK in human eosinophils [21,22]. In this study we showed that JNK is slowly activated during constitutive human eosinophil apoptosis and the same tendency can be seen in dexamethasone-induced apoptosis. However, we did not observe any increased activity of JNK in dexamethasone-treated eosinophils as compared with untreated cells. This is in line with our results showing that JNK inhibitors do not reverse the effect of steroids. In this study we also showed that serine 63-phosphorylated c-Jun can be detected in freshly isolated human eosinophils, which reflects the activation of JNK [27]. However, no further activation (i.e. phosphorylation of serine 63 residues) of c-Jun could be observed although JNK was found to be slightly activated. This can be explained by the fact that activities of c-Jun and JNK can be separately regulated apart from phosphorylation of serine 63 and 73 residues of c-Jun that is strictly dependent of nuclear JNK activity [27]. JNK activation and even nuclear translocation does not inevitably result in phosphorylation of c-Jun since JNK also catalyzes the phosphorylation of other nuclear substrates [27]. In addition, JNK can associate with c-Jun through multiple contacts which do not require the N-terminal phosphorylation of the serine residues 63 and 73 [27].

Addressing the question of the specificity of the JNK inhibitors, which is essential when drawing conclusions from studies made with protein kinase inhibitors, Bain and co-workers [28] have recently shown that the most commonly used JNK inhibitor SP600125 is a relatively weak and non-specific inhibitor of JNK. To further complicate the studying of signalling cascades associated with protein kinases, the use of transfection techniques to either block or enhance certain signalling pathways in non-dividing and rapidly dying cells like eosinophils is by large impossible. To overcome these problems, we used another pharmacological approach to inhibit JNK. L-JNKI1 is novel cell-permeable inhibitor of JNK that is coupled to the HIV transcription factor TAT, which acts as a protein transduction domain (PDT) and rapidly delivers the peptide into the cell [25,26,29]. Even though there exist...
some limitations to the use of protein transduction domains, their low toxicity and ability to deliver to at least some primary non-dividing cells make PDTs an attractive tool for both in vitro and in vivo studies [29]. Based on our observations, L-JNKI1 can be applied to studies in eosinophils. For example, we have recently shown that nitric oxide induces activation of JNK and reverses IL-5-mediated human eosinophil survival by inducing apoptosis, which can be inhibited by L-JNKI1 [23].

Recently, based on studies with sensitized animals, a role for the JNK in chronic allergic airway inflammation and remodeling associated with bronchial hyperresponsiveness has been proposed [30]. Pretreatment with the JNK inhibitor SP600125 has been shown to reduce the numbers of inflammatory cells, including eosinophils in the bronchoalveolar lavage fluid after allergen challenge in mice and rats [30–32]. Interestingly, the numbers of eosinophils in the bronchial tissue were not reduced after pre-treatment with SP600125 [32]. Our present result that inhibition of JNK activity does not increase but rather decreases apoptosis of eosinophils is in line with the inability of SP600125 to reduce tissue eosinophilia. However, SP600125 reduces the production of several cytokines and chemokines [30–32] as well as inhibits several other kinases [28] and thus the net effect of SP600125 on pulmonary inflammatory indices is a result of a combination of all its effects. However, based on our present results and those reported from animal studies [32], inhibition of JNK activity may not be an optimal target to reduce lung tissue eosinophilia.

In conclusion, we have in the present study demonstrated that JNK mediates spontaneous apoptosis of...
human eosinophils. In contrast, our results suggest that JNK may not be responsible for the enhancement of eosinophil apoptosis by glucocorticoids.

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