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JNK mediates TGF-β1-induced epithelial mesenchymal transdifferentiation of mouse transformed keratinocytes

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Abstract In this study we analyzed the role of the c-Jun N-terminal kinases (JNK) pathway in the TGF-β1 stimulation of urokinase-type plasminogen activator (uPA), initial stages of epithelial-mesenchymal transdifferentiation (EMT) and cell migration. TGF-β1 induces JNK phosphorylation, c-Jun transactivation and AP1 activation. The involvement of JNK was evaluated using dominant negative mutants SEK-1 AL, JNK and cJun, depletion of JNK1,2 proteins by treatment of cells with antisense oligonucleotides, as well as the chemical inhibitor SP600125. Our results demonstrated that the JNK pathway is required in the TGF-β1 enhancement of uPA, fibronectin, E-cadherin delocalization, actin re-organization and vimentin expression, concomitant with the induction of cell migration. These results allow us to suggest a role of JNK in the TGF-β1 induction of EMT in relation with the stimulation of malignant properties of mouse transformed keratinocytes.

Keywords: JNK; TGF-β1; uPA; Keratinocytes; EMT

1. Introduction

The process of epithelial-mesenchymal transdifferentiation (EMT) is characterized by a set of transient phenotypic changes often associated with the acquisition of migratory properties by cancer cells and provides a means for cancer propagation through out the organism [1,2].

The TGF-β super-family is implicated in the regulation of cell proliferation, differentiation, migration, extracellular matrix production, apoptosis and tumorigenesis [3]. TGF-β binds to the functional complex of the TGF-β family of receptors at the cell surface [4,5], which, in turn, activate the Smads and MAP kinases pathways including Ras, Erk1/2, and JNK1 [6–12]. Transforming growth factor-β1 (TGF-β1) has been postulated to have a dual role in tumour progression by acting as tumor suppressor in the early stages of carcinogenesis, and as pro-oncogenic in the last stages of metastatic disease [13,14]; it also induces EMT of transformed cells [15,16].

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Abbreviations: EMT, epithelial-mesenchymal transdifferentiation; JNK, cJun N-terminal kinase; uPA, urokinase type plasminogen activator; TGF-β1, Transforming growth factor-β1; Fn, Fibronectin; E-cad, E-cadherin; vim, vimentin

TGF-β1 increases plasminogen activator like-urokinase (uPA) expression, which is regulated at the transcriptional level by the AP-1 transcription factor [17,18]. The transcription factor c-Jun (AP-1 component) is consequently activated by the c-Jun NH_2-terminal kinase (JNK) [19].

Although Ras-MAP kinases and the Smad signal pathways contribute to malignant enhancement by TGF-β1 [10,20,21], the mechanisms that mediate TGF-β1 transformed cell responses have not been fully elucidated. In the present study, we have evaluated the role of the JNK pathway in the stimulation of uPA, cell migration and EMT by TGF-β1. We found that TGF-β1 activates JNK MAP kinase and transactivates the c-Jun and AP1 complex. In addition, the inhibition of the JNK pathway was found to affect uPA and cell migration and consequently the initial step of malignant EMT of mouse skin transformed cells.

2. Material and methods

2.1. Cell cultures and treatment conditions

The PDV cell line [22] was cultured as described [10]. Cells were incubated with TGF-β1 (Calbiochem-Novabiochem, La Jolla, CA) at a final concentration of 10 ng/ml for the indicated period of time. The chemical JNK inhibitor SP600125 (10 μM) provided by Calbiochem was added 30 min before the addition of TGF-β1.

2.2. Plasmids

AP-1-Luc and p-c-Fos-luc were provided by Dr. A. Corbí (Centro de Investigaciones Biológicas, Madrid, Spain), GAL4-c-Jun was provided by Dr. JL. Jameson (Northwestern University, Chicago, IL). Vector pFA-Luc (5× GAL4-binding element) was purchased from Stratagene. The pcDNA3.1 blank vector was obtained from Invitrogen (Carlsbad, CA). The p-4.8 uPa-Luc luciferase reporter plasmid (−4.8 kb of murine uPA promoter) was provided by Dr. P Munoz-Canoves (Center for Genomic Regulation (CRG), Barcelona, Spain). Dominant negative SEK1 AL (MEK4 mutant) was kindly provided by Dr. J. R. Woodgett (York University, Toronto, Ontario, Canada). Dominant Negative JNK and cJun were kindly provided by J. M. Redondo (Instituto Severo Ochoa, UAM, Madrid, Spain).

2.3. Antibodies

The anti-phospho JNK monoclonal antibody, anti-JNK1 rabbit polyclonal antibody, anti-p38 and anti-fibronectin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-cadherin-E rat monoclonal antibody was kindly provided by Dr. M. Quintanilla (Instituto de Investigaciones Biomédicas, Madrid, Spain). The anti-vimentin and anti α-tubulin monoclonal antibody (Sigma, St. Louis, Mo).

2.4. Immunofluorescence

Cells seeded on coverslips were fixed with 4% *p*-formaldehyde for 10 min at room temperature. For JNK1,2, F-actin and vimentin immu-

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nostaining cell monolayers were permeabilized with 0.1% Triton-X 100 for 2 min. at room temperature. F-actin was stained using Phalloidin-Alexa Fluor (Molecular Probes, Eugene, OR), and secondary rabbit, mouse and rat antibodies were coupled to FITC (Sigma, St. Louis, Mo).

2.5. Immunoblotting

Proteins were separated by SDS–PAGE and then transferred to nitrocellulose membranes (BIORAD, Hercules, CA) which were blocked in 4% milk (diluted in Tris-buffered saline and 0.5% Tween 20) and incubated with the appropriate antibody at 4 °C overnight. The targeted proteins were detected by enhanced chemiluminescence as indicated by the manufacturers (Pierce).

2.6. Transient transfections and reporter gene measurements

For luciferase assays, PDV cells were transfected with superfect (Quiagen) following the manufacturer's instructions. Typically, 2×10^5 cells were plated in each well of a 24-well plate. The next day cells were transfected with 500 ng/well of each specific luciferase construction, together with 25 ng/well SV40-β-Gal RL (Promega) as internal control for transfection efficiency. After 24 h of TGF-β1-treatment cells were lysed and luciferase activity determined. For the GAL4 fusion transactivation luciferase determinations, cells were co-transfected with 0.5 μg of pFA-Luc and 0.2 μg of Gal4-cJun1.

2.7. Oligodeoxynucleotide treatments

JNK1 antisense (TCACGCTTGCTTCTGCTCAT) and JNK2 antisense (TCACATTTACTGTCGCTCAT) phosphorothioate-modified oligodeoxynucleotides [36] were synthesized and purified by Isogen Bioscience BV (Maarssen, The Netherlands). As control, scrambled S-oligo were used. A 1:1 mixture of antisense oligonucleotides for both JNK 1 and 2 was added to the cells (50% confluent) and treated as described by Santibanez et al. [10]. The media containing oligos was changed daily.

2.8. Zymographic and migration assay

The uPA secreted activity of cell cultures was determined by caseinolytic zymography and the migration by wounded assay as previously described [10].

3. Results

3.1. TGF-β1 induces JNK activation and c-Jun and AP1 transactivation in PDV cells

To examine whether TGF-β1 activates the JNK pathway, we performed JNK phosphorylation, AP1 and cJun transactivation assays. TGF-β1 increased JNK phosphorylation within 60 min. (Fig. 1A), reached its maximum (~5.0 fold time) at 120 min and started to decrease after 4 hours. This rapid and

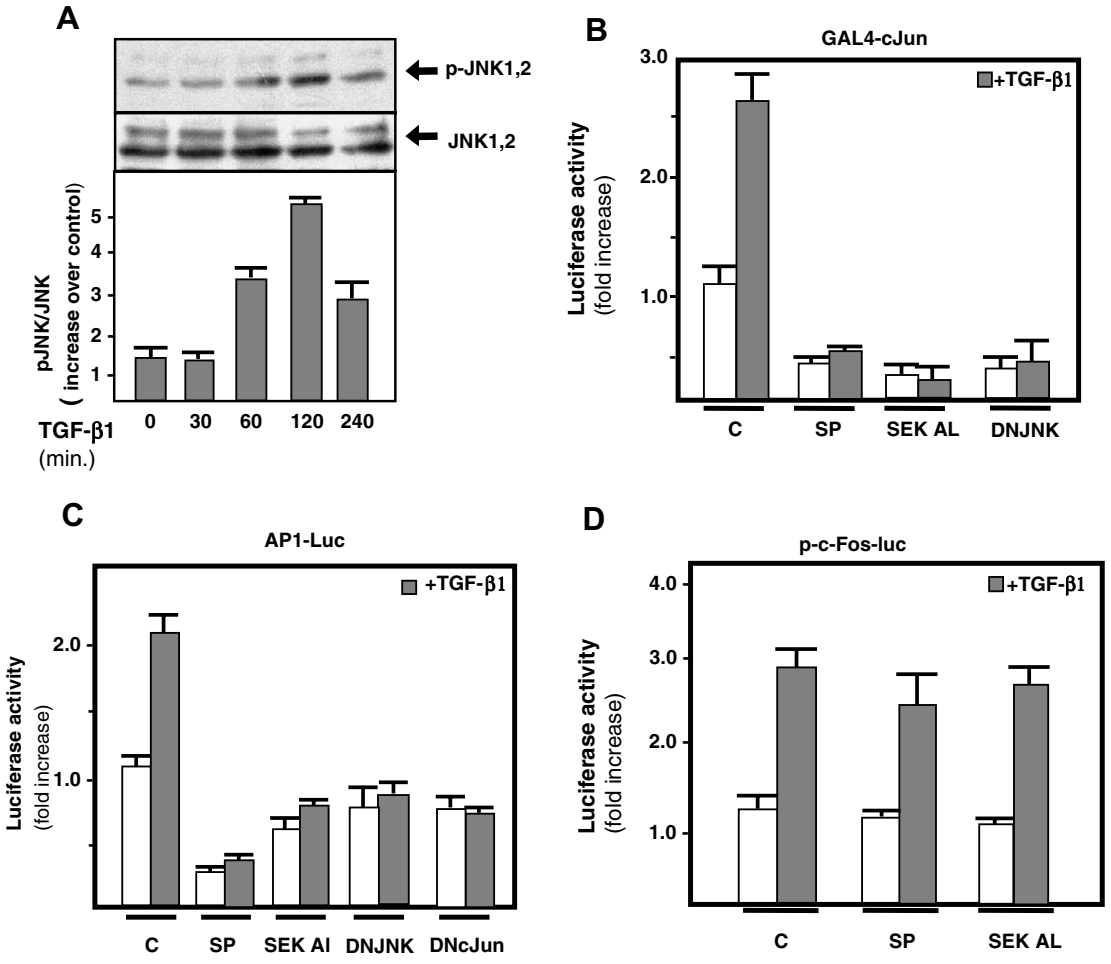

Fig. 1. TGF-β1 induces on JNK Phosphorylation, AP1 and c-Jun transactivation in PDV cells. (A) Cell lysates were immunobloted and revealed with either anti-p-JNK or JNK antibodies. Two independent experiments were performed and a representative is shown. Bottom part: densitometric scans of results shown in top part. Cells were transiently transfected with Gal-4-cJun/pRf-Luc (B), AP1-luc (C) or c-Fos-luc (D). Dominants negative SEK-AL, JNK or cJun were co-transfected, or pre-treated with JNK inhibitor SP600125 as in indicated points. Transfections and assays were performed independently three times, each run in triplicate.