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p38 MAP kinase enhances EGF-induced apoptosis in A431 carcinoma cells by promoting tyrosine phosphorylation of STAT1

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ABSTRACT

While epidermal growth factor (EGF) is a well known mitogen, high doses of EGF result in a paradoxical apoptotic response in the cells that overexpress EGF receptor such as A431 epidermoid carcinoma cells. EGF-induced apoptosis in A431 cells is dependent upon activation of transcription factor STAT1. In this study, we demonstrate that p38 MAP kinase is another important mediator of EGF-dependent pro-apoptotic response in A431 cells. By utilizing p38 MAP kinase inhibitors, SB203580 and BIRB0796, we significantly reduced the integral growth-inhibiting as well as pro-apoptotic effects of EGF. Moreover, we observed that inhibition of p38 MAP kinase markedly decreased phosphorylation of tyrosine 701 in STAT1, while neither EGF-induced accumulation nor serine phosphorylation of STAT1 was decreased. We propose that p38 MAP kinase mediates STAT1 tyrosine phosphorylation, thereby enforcing EGF-induced apoptosis.

1. Introduction

Epidermal growth factor (EGF)[2] binds EGF receptor (EGFR), one of transmembrane tyrosine kinases receptors. It activates multiple signaling pathways such as PI-3 kinase, phospholipase Cγ, mitogen-activated protein kinase (MAPK), and STAT pathways [1,2]. Various elements of the EGF-induced signaling cascade stimulate cell cycle progression and cell survival, however there are notable exceptions. Namely, nanomolar concentrations of EGF invoke either anti-proliferative or even an apoptotic response in some EGFR-overexpressing cancer cell lines such as A431 squamous epidermoid carcinoma cell line [3,4]. Oncogenic transformation of such cells is dependent upon EGFR [5], therefore, EGF treatment might appear selective and sustainable instrument in anti-cancer therapy.

The transcription factor STAT1 is required for EGF-induced apoptosis in A431 cells [6,7]. Upon phosphorylation of the tyrosine at position 701 of STAT1, the protein translocates to the nucleus subsequently inducing transcription of a number of genes [8]. Various kinases, e.g. MAP kinases (MAPK), have been found to play a role in STAT1 regulation [8].

The MAP kinases function as critically important molecules in controlling growth, proliferation, differentiation, and survival in many cell types [9]. A member of MAP kinases, p38 MAPK, is often activated by cellular stress and triggers an apoptotic response [10]. It is also known to enhance the activity of STAT1 through multiple mechanisms [11–13]. p38 MAPK has been shown to be activated upon EGF stimulation in A431 cells, with some authors suggesting that it might mediate EGF-induced apoptosis [14]. Tikhomirov and Carpenter [15] have shown the significance of p38 MAPK in cell death response to EGF in cells overexpressing both EGFR and ErbB2. And yet the same year Morazzani and colleagues [16] announced the importance of p38 MAPK for suppression of EGF-induced apoptosis in A431 cells. This apparent contradiction compelled us to investigate the matter further.

2. Materials and methods

The manuscript was written in accordance with Uniform Requirements for manuscripts submitted to Biomedical journals.

2.1. Reagents

All reagents except indicated otherwise were from Sigma–Aldrich (St. Louis, MO, USA). Plastic dishes and plates were from Nalge Nunc International (Neerijse, Belgium) or Corning Life Sciences (Lowell, MA, USA). Cell culture reagents from Paneco (Moscow, Russia) and serum from PAA Laboratories (Pasching, Austria) were used. The inhibitors of MEK1 (PD98059), JNK (SP600125), p38 MAPK (SB203580) were from Calbiochem (La Jolla, CA, USA). The other inhibitor of p38 MAPK, BIRB0796, was from Axon Medchem, Groningen, The Netherlands. Nitrocellulose

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[2] *Abbreviations:* EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, MAP kinase, mitogen-activated protein kinase.

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membranes and electrophoretic equipment were from Bio-Rad Laboratories (Hercules, CA, USA). Human recombinant EGF was from PanBiotech GmbH (Aidenbach, Germany). Primary antibodies were rabbit polyclonal from Cell Signaling Technology, Danvers, MA, USA, the exceptions are indicated below. Antibodies against the following epitopes were used: active (Asp175-cleaved) caspase 3, p38 MAPK, phospho-p38 MAPK(Thr180/Tyr182), phospho-MK2(=MAPKAPK2)(Thr334), STAT1 (monoclonal, BD Transduction Laboratories), phospho-STAT1(Tyr701), phosphor-STAT1(Ser726), tubulin (Sigma–Aldrich, monoclonal, clone B-5-1-2), GAPDH. Secondary antibodies for Western blotting were from Cell Signaling Technology and Sigma–Aldrich, and ECL substrates (Western Blotting and SuperSignal West Femto) were form Pierce (Rockford, IL, USA).

2.2. Cell culture and treatments

A431 cells were from ECACC (No. 85090402). The cells were cultivated in DMEM supplemented with 10% fetal calf serum and 20 mg/ml gentamycin at 37 °C, 5% CO_2. For all the experiments, the cells were plated at a density of 1.1×10^4 cells in 0.18 ml media per cm^2. 16–24 h post seeding the media was changed to fresh media containing one of kinase inhibitors where it was necessary (50 μM PD98059, 5 μM SP600125, 5 μM SB203580, or 5 μM BIRB0796). EGF (30 or 50 ng/ml) was added 30 min post inhibitor treatment where it was necessary. The time of EGF addition was considered zero reference time. There were no further changes of the medium or EGF additions.

2.3. Western blotting

To prepare total lysates, the cells were rinsed twice with phosphate-buffered saline (PBS), lysed on ice with PBS containing 1% Triton X-100, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitors cocktail (Sigma, P8340, dilution 1:300) for 10 min, and cleared at 15,000*g* for 15 min. For active caspase 3 staining, the floating cells were collected and lysed together with the attached cells. Protein content was detected using Bradford method. Disc-SDS polyacrylamide electrophoresis, transfer, immunostaining, and ECL detection were carried out according to standard protocols of Bio-Rad Laboratories, Pierce, and antibody manufacturers.

2.4. MTT assay

Media from cells grown in a 96-well format was aspirated. 50 μl thiazolyl blue tetrazolium bromide (MTT, 0.715 mg/ml in serum-containing growth medium) was added to the wells. After 2 h at 37 °C, 5% CO_2 the solution was changed to 50 μl DMSO. The plates were shaken at room temperature for 2 min. Absorbance was measured at 570 nm using a Fluorofot «Charity» reader (Probanauchpribor, S.-Petersburg, Russia). Average absorbance of five independent readings at a given time point was normalized to the average absorbance at the time of EGF addition. The average data of three to four independent experiments and the corresponding standard errors of average are presented.

2.5. Microscopy

Cells were analyzed using Carl Zeiss Axiovert 40 microscope in phase-contrast or fluorescent mode. For DAPI staining the floating cells were pelleted by centrifugation. The adherent cells were detached with chymotrypsin–EDTA solution, combined with the floating cells fraction, fixed by direct addition of 37% formaldehyde to the media for a final concentration of 4%, washed once with PBS, and mounted in 50% glycerol/PBS containing 1 μg/ml DAPI. The percentages of apoptotic nuclei were calculated. The average data of three independent experiments and the corresponding standard errors of average are presented. Student's *T*-test was utilized as a test of significance.

3. Results

EGF in nanomolar concentrations has been long known to have a growth-inhibitory effect on A431 cells [3]. We tested the influence of different MAPKs on EGF-induced growth inhibition by MTT assay (Fig. 1A) that allowed us to estimate the number of viable cells by measuring mitochondrial dehydrogenase activity. EGF itself greatly diminished the viable cell number as expected: we observed cell death instead of cell proliferation. Inhibition of ERK MAPK cascade with PD98059 even slightly pronounced the effect of EGF. In contrast, p38 MAPK inhibition with SB203580 significantly decreased EGF-induced effect, causing stabilization of the number of viable cells. JNK MAPK blocking with SP600125 also diminished EGF-induced growth inhibition, but the effect was weak. While the specificity of SP600125 is known to be low [17], its action may be due to the interruption of other signaling pathways, e.g. p38 MAPK. Blocking of p38 MAPK also decreased EGF-induced changes in cellular morphology, such as cell rounding and detachment from substrate (Fig. 1C).

To verify that the effect of p38 MAPK inhibitor, SB203580, was specific to p38 MAPK, we used another p38 MAPK inhibitor with an unrelated chemical structure, BIRB0796, as recommended by Bain and colleagues [17]. The observed effect was similar to the effect of SB203580, partially preventing EGF-induced decrease in cell number (Fig. 1B), and thus demonstrating that p38 participates in the integral growth inhibition in A431 cells triggered by EGF.

Activating phosphorylation of p38 MAPK under our experimental conditions was confirmed by Western blot analysis (Fig. 1D). Our findings demonstrate the two-wave dynamics of p38 MAPK activation, with p38 MAPK phosphorylation high at 10 min post EGF stimulation and a subsequent decrease followed by an increase again at 24 h. Phosphorylation of MAPKAPK2, a natural substrate of p38 MAPK, followed the same trend. As expected, SB203580 inhibited phosphorylation of MAPKAPK2.

We have previously shown apoptosis to be the major mechanism of the integral growth-inhibitory effect of EGF in A431 cells under our experimental conditions [7], so we checked the influence of p38 MAPK on apoptosis. A decrease in the number of cells presenting apoptotic nuclear morphology (DAPI staining) upon blocking p38 MAPK demonstrated strong suppression of EGF-induced apoptosis (Fig. 2A and B). The blocking of p38 MAPK also decreased the amount of active cleaved caspase 3 in the cells (Fig. 2C). These findings demonstrate that p38 MAPK is a critical component of the pathway leading to apoptosis in A431 cells upon EGF treatment.

One possible mechanism by which p38 MAPK could influence EGF-induced apoptosis is through the impact on the transcription factor STAT1, the main mediator of EGF-induced apoptosis. Since STAT1 protein itself is shown to be accumulated upon EGF stimulation of A431 cells [7], at first we checked whether SB203580 blocks STAT1 accumulation. However, SB203580 appeared even to increase the rate of accumulation of STAT1 (Fig. 3). Following this finding we then looked at the phosphorylation of STAT1. p38 MAPK is known to be able to directly phosphorylate serine 727 of STAT1, thus enhancing the activity of STAT1 [8,11]. However, in A431 cells treated with EGF the level of Ser727-phosphorylated STAT1 was consistent with total STAT1 protein content and apparently was not affected by p38 MAPK inhibitor SB203580 (Fig. 3). Nonetheless, the amount of Tyr701-phosphorylated STAT1 did decrease under conditions of p38 MAPK inhibition (Fig. 3). Since tyrosine phosphorylation is the main cause of STAT1 activation, and

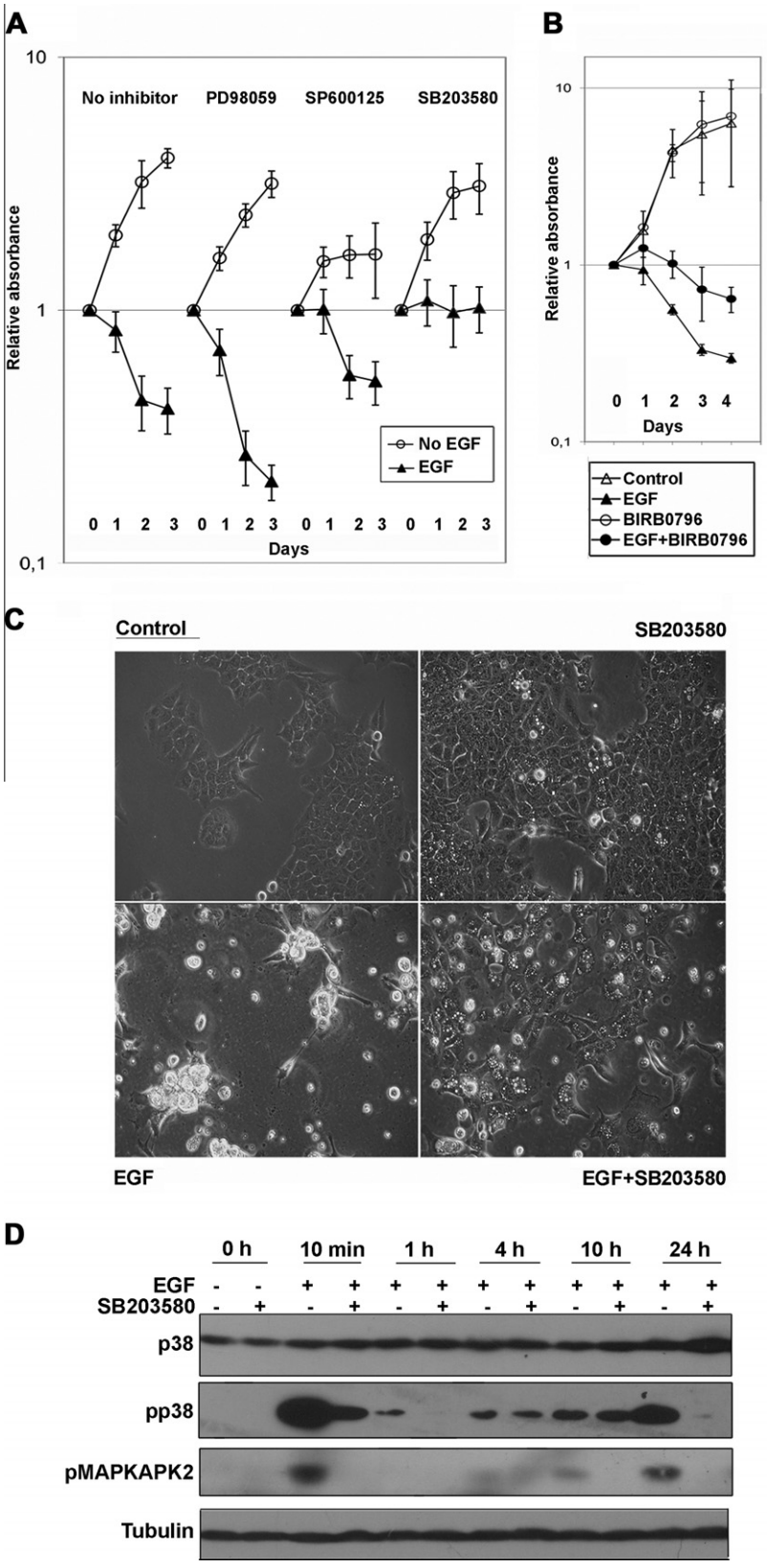

Fig. 1. p38 MAPK participates in EGF-induced integral growth inhibiting response in A431 cells. (A) The cells were pretreated with different MAPK inhibitors (*PD98059*, an inhibitor of ERK pathway, *SP600125*, an inhibitor of JNK, *SB203580*, an inhibitor of p38) or left untreated, stimulated with *EGF* where shown, and analyzed daily by MTT assay. Average relative absorbances and standard errors of average are presented. (B) The cells were pretreated with the other p38 MAPK inhibitor (*BIRB0796*) or left untreated, stimulated with *EGF* where shown, and analyzed daily by MTT assay. (C) The cells were pretreated with the p38 MAPK inhibitor (*SB203580*) or left untreated, stimulated with *EGF* where shown, and grown for 3 days. Images were made by utilizing phase-contrast microscopy technique. (D) The cells were pretreated with *SB203580*, incubated with or without *EGF* for indicated times, and then analyzed by Western blotting.

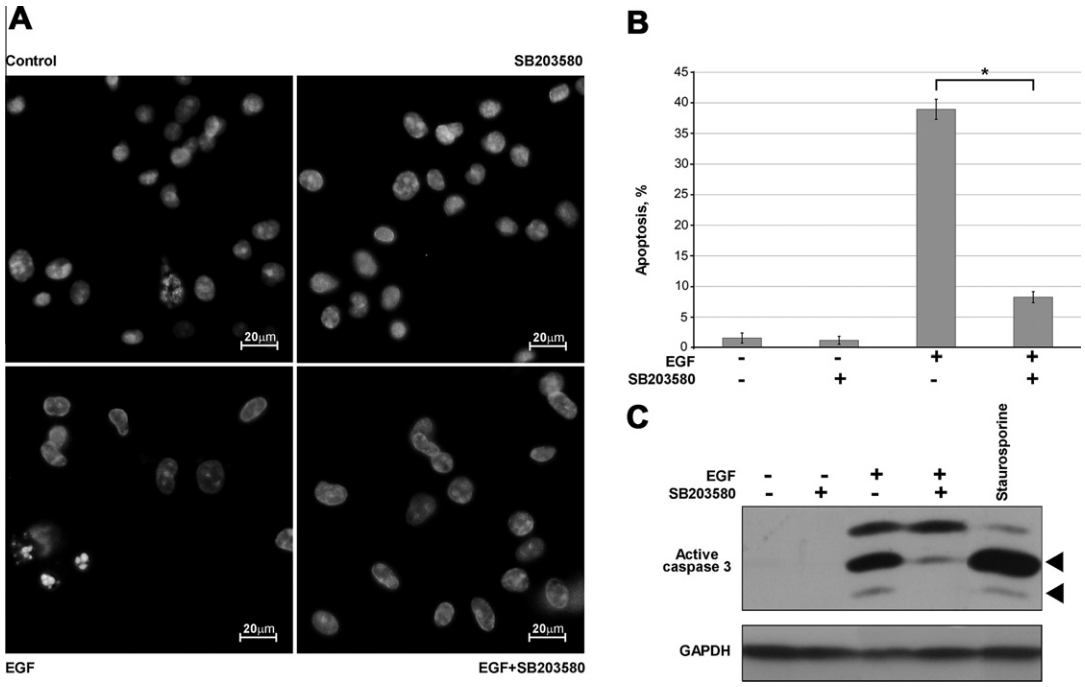

Fig. 2. p38 MAPK enhances EGF-induced apoptosis in A431 cells. (A) The cells were pretreated with *SB203580* or left untreated, incubated with or without *EGF* for 2 days, and the morphology of the nuclei was analyzed by DAPI staining. The percentages of apoptotic nuclei and the standard errors of average were calculated.*$p < 0.01$. (B) Examples of microscopic images of DAPI-stained cells that are quantified in (A). (C) The cells were treated as in (A) and analyzed by Western blotting. Active caspase 3 is marked with arrowheads. The upper band is non-specific as described in the antibody manufacturer's manual.

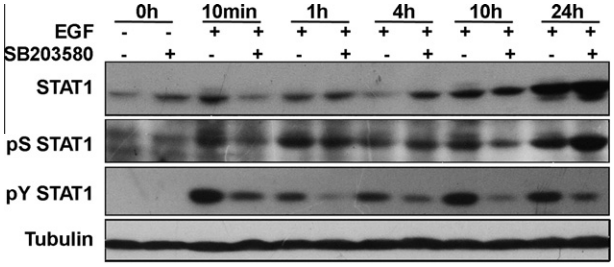

Fig. 3. Inhibition of p38 MAPK brings down tyrosine phosphorylation of STAT1 in spite of increasing STAT1 protein content. The A431 cells were pretreated with *SB203580* or left untreated, incubated with or without EGF for indicated times, and then analyzed by Western blotting.

STAT1 itself is essential for EGF-induced apoptosis in A431 cells [7], we conclude that promoting STAT1 tyrosine phosphorylation is at least one of mechanisms of the pro-apoptotic effect of p38 MAPK in this system.

4. Discussion

p38 MAPK is known to mediate pro-apoptotic signals from various extracellular stimuli [10]. Tikhomirov and Carpenter [15] observed p38-dependent apoptosis in different cell lines where EGFR and its heterodimerization partner, ErbB2, were simultaneously overexpressed. In addition, Song et al. [14] hypothesized about the importance of p38 MAPK in EGF-induced apoptosis in A431 cells, though solely on the basis of EGF-induced activation of p38. Our data expand these findings, demonstrating the significant role of p38 in EGF-induced apoptosis in A431 cells and providing the mechanism of its action.

On the contrary, Morazzani and colleagues [16] reported that inhibition of p38 MAPK amplified EGF-induced apoptosis in A431 cells via reducing the content of $\alpha 2\beta 1$-integrin, thus causing the cells to form an EGF-sensitive monolayer (2D-population) instead of aggregates (3D). However, despite their data about p38-dependent integrin induction and cell aggregation being accurate and convincing, it appears to us that no direct evidence of p38-dependent cell survival was provided in their study. On the other hand, the contradiction may also be due to different sources of A431 cells and thus to certain diversity between the cells used, the phenomenon that we can attest to on the base of our own vast experience with this highly variable cell line.

Various cases of how p38 MAPK enhanced STAT1 signaling have been reported. p38 MAPK often phosphorylates Ser727 in STAT1 in response to stress, resulting in enhanced activation of STAT1-dependent transcription [8,11]. However, this happens neither upon interferon γ treatment [11,12] nor upon EGF treatment (Fig. 3). Ramsauer and colleagues reported that anisomycin-activated p38 MAP kinase enhances interferon γ-activated STAT1-dependent transcription even when Ser727 is mutated (unfortunately, they had not verified Tyr701 phosphorylation) [12]. Also, the necessity of p38 MAPK for STAT1 tyrosine phosphorylation was reported in hyperosmolarity-treated cells [13]. Here we present the novel connection between p38 MAPK and the activation of the transcription factor STAT1 upon EGF stimulation.

p38 MAPK is a serine-threonine kinase and cannot phosphorylate Tyr701 in STAT1 directly. It is generally accepted that upon EGF treatment STAT1 is first recruited to the intracellular domain of activated EGF receptor and there is it tyrosine phosphorylated by Src family kinases or by EGFR itself [2,8]. The role of p38 MAPK for providing STAT1 tyrosine phosphorylation must be either in enhancement STAT1 recruitment or in increasing the ability of tyrosine kinases to phosphorylate STAT1. p38 MAPK has been found to phosphorylate multiple serine and threonine sites on EGFR directly [18,19] and to alter the degree of phosphorylation of several tyrosine residues on EGFR [20], which may lead to enhancement of STAT1 activity.

Since p38 MAPK is a well known pro-apoptotic molecule [10], we are unable to rule out its STAT1-independent role in EGF-driven apoptosis induction. However, the crucial role of STAT1 in this system, as well as the remarkable dependence of STAT1 tyrosine phosphorylation on p38 MAPK activity makes us speculate that p38 MAPK acts mainly through STAT1.

In conclusion, we would like to note that as far as EGF-induced apoptosis is almost entirely limited to some types of cancer cells [21], we hope that understanding of its mechanism can help in development of new highly targeted ways of treatment of these cancers.

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