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Effects of MAP Kinase Inhibitors on Epidermal Growth Factor-Induced Neoplastic Transformation of Human Keratinocytes

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Abstract

We previously reported data regarding the mechanism of neoplastic transformation in JB6 Cl41 mouse skin epidermal cells. However, experimental *in vitro* models for studying neoplastic transformation of human cells could provide further insight into the mechanisms of human cancer development. In this study, we have established a neoplastic transformation model with HaCaT cells, a human keratinocyte cell line, and showed the usefulness of this cell line for studying the mechanisms of neoplastic transformation. Epidermal growth factor (EGF) treatment induced a dose-dependent anchorage-independent cell transformation in HaCaT cells. Furthermore, PD98059, a MAP kinase/ERK kinase (MEK) inhibitor, or SP600125, c-Jun N-terminal kinase (JNK) inhibitor, decreased cell growth, EGF-induced DNA synthesis and transformation. Unlike observations in the JB6 mouse epidermal cell model, SB203580, a stress-activated protein kinase-2/p38 α and β (p38) inhibitor, increased EGF-induced transformation in HaCaT cells. These results suggest that extracellular-signal regulated kinase (ERK), JNK or p38 are implicated in EGF-induced neoplastic transformation of human cells.

Keywords

EGF; cell transformation; human keratinocyte; MAP kinase

Abbreviations

BME, basal medium Eagle; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal regulated kinase; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase

INTRODUCTION

The epidermal growth factor (EGF) receptor is overexpressed in tumors such as lung, colon, kidney and head and neck carcinomas [1,2]. We have previously published reports regarding the mechanism of EGF-induced neoplastic transformation in JB6 Cl41 mouse skin epidermal cells [3–6]. This model is a well-established culture system for studying neoplastic transformation [7]. However, the applicability of mouse data to the human situation is not always straightforward. Experimental *in vitro* models using human cells for studying neoplastic transformation might provide further insight into the mechanisms of carcinogenesis.

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Human cells have a remarkable resistance to transformation by oncogenes and chemical or physical carcinogens, possibly explaining why no reproducible or quantitative transformation studies have been performed so far [8,9]. To study genetic and phenotypic changes involved in the process of human skin carcinogenesis, Fusenig et al. [8] developed the spontaneously immortalized keratinocyte cell line, HaCaT, which was derived from normal adult human skin, and developed during prolonged cultivation at a reduced Ca^{2+} concentration and elevated temperature [10]. Unlike human papilloma virus-immortalized keratinocytes [12], HaCaT cells escape cellular senescence *in vitro* but remain non-tumorigenic over extended culture periods when maintained under standard conditions [11]. Despite their altered and unlimited growth potential, HaCaT cells, similar to normal keratinocytes, reform an orderly structured and differentiated epidermal tissue when transplanted into nude mice [10]. The major difference between normal and HaCaT cells is the fact that HaCaT cells express mutations in both alleles of the p53 gene [13,14] and have lost chromosome 3p [11]. Although the exact mechanism of spontaneous immortalization still remains to be elucidated, the loss of one or more senescence genes on chromosome 3p is thought to be a major event in the immortalization of the HaCaT cells [15].

By transfection with the *ras* oncogene, HaCaT cells can be transformed into a tumorigenic state [16,17]. Ras proteins function as key regulators of cellular signal transduction pathways [18,19]. Diverse extracellular stimuli that promote cell proliferation and differentiation also activate cell surface receptors, which converge on and promote the transient activation of Ras [18,19]. This genetically activated model has provided useful knowledge regarding cellular signal transduction pathways. However, whether a specific ligand, like a growth factor, can induce transformation in HaCaT cells is not clear. If a tumor promoter can induce transformation in HaCaT cells, this system can provide a very useful and convenient model for studying the human transformation mechanism and screening of potential chemopreventive agents.

Epidermal growth factor (EGF) is known to activate mitogen activated protein (MAP) kinases through Ras activation. MAP kinases are central components involved in transducing signals elicited by growth factors and stress-related stimuli. In mammalian cells, the three best-characterized MAP kinase pathways are the extracellular-signal regulated kinase (ERK), stress-activated protein kinase-1/c-Jun N-terminal kinase (JNK) and stress-activated protein kinase-2/p38 α and β (p38) pathways [20]. ERK is mostly activated by growth factors and cytokines and is especially involved in transducing proliferation signals and in promoting cell survival by conferring protection against cell death [21,22]. JNK and p38 are also sensitive to growth factors, but they are more specifically recognized as stress-responsive pathways. These pathways are implicated in inducing a homeostatic response against stress and, depending on the cellular context, are important positive or negative modulators of the apoptotic cell death program [20].

In this study, we have established a neoplastic transformation model of human cells, and showed that pharmacological inhibition of ERK, JNK or p38 affect EGF-induced transformation in HaCaT cells. These results provide further insight into the mechanisms of human cancer development.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Basal medium Eagle (BME) was obtained from Sigma (St. Louis, MO). Epidermal growth factor (EGF), PD98059, a MEK inhibitor [23,24], SP600125, a JNK inhibitor [25], and SP203580, a p38 inhibitor [26], were from Calbiochem-

Novabiochem corp. (San Diego, CA). Fetal bovine serum (FBS) was from Gemini Bio-Product (Calabasas, CA). The antibody against phospho-p38 was from BD Biosciences (Palo Alto, CA). Antibodies against p38, phospho-p44/42 MAPK (phospho-ERK), p44/42 MAPK (ERK), phospho-JNK, JNK, phospho-c-Jun (Ser63) and c-Jun were from Cell Signaling Technology Inc. (Beverly, MA).

Cell Culture

HaCaT cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and glutamine (2 mM) at 37 °C in a humidified atmosphere of 5% CO₂.

Anchorage-Independent Transformation Assay

The role of MAP kinases in EGF-induced cell transformation was investigated in HaCaT cells. Cells (8000 cells) were exposed to EGF (0.05 to 10 ng/ml) with or without different concentrations of various MAP kinase inhibitors in 1 ml of 0.33% BME agar over 3 ml of 0.5% BME containing 10% FBS. The cultures were maintained in an incubator at 37 °C and 5% CO₂ for 14 days, and the cell colonies were scored using a microscope and the Image-Pro PLUS computer software program (Media Cybernetics; Silver Spring, MD) as described by Colburn et al [27].

Immunoblotting

Immunoblotting was performed following the instructions from Cell Signaling Technology Inc. In brief, HaCaT cells were cultured to 80% confluence and then starved in serum free DMEM for 30 to 36 h in an incubator at 37 °C and 5% CO₂, and the cells were treated with EGF for various times as indicated. The cells were lysed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) and harvested immediately. The lysed samples were transferred to microcentrifuge tubes and sonicated for 10 to 15 seconds, and then heated for 5 min at 95 °C. The samples were loaded on SDS-polyacrylamide gels for electrophoresis and subsequently transferred onto polyvinylidene fluoride membranes (Millipore; Bedford, MA). After blocking, the membranes were incubated with the specific primary antibody with gentle agitation overnight at 4 °C. After washing, the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Detection was performed using the ECL Plus Western Blotting Detection reagents (Amersham Biosciences Corp.; Piscataway, NJ).

[³H]-Thymidine Incorporation Assay

HaCaT cells (1×10⁴ cells) were seeded in 96-well plates. After culturing for 24 h, media were replaced with 0.05% FBS/DMEM and cells were incubated for 24 h. HaCaT cells were incubated with PD98059, SP600125 or SB203580 at various concentrations or their vehicle, 0.1% DMSO, as a control, for 1h. Then, HaCaT cells were treated with 10 ng/ml EGF. After culturing 18 h, [³H]-thymidine (0.5 µCi/well) was added to each well, and then cells were incubated for another 6 h. After harvesting of cells, the incorporation of the [³H]-thymidine was measured by liquid scintillation counting (LS6500, Beckman Coulter Inc.; Fullerton, CA).

Statistics

Statistical analysis to find significant differences between groups was carried out using the Student's t test.

RESULTS

EGF Induces Transformation in HaCaT Cells

To study genetic and phenotypic changes involved in the process of human skin carcinogenesis, Fusenig et al. [8] developed the immortalized keratinocyte cell line, HaCaT. Although colony-forming efficiency in agar constantly increased with subsequent passages, anchorage-independent colony-formation is reported to be very low in HaCaT cells [15]. Actually, the colony-forming capacity without tumor promoter treatment in HaCaT was almost negligible in our results (42.8 ± 37.7 colonies/8000 cells, $n = 3$, Figure 1). Although transfection with the *ras* oncogene can transform HaCaT cells into an anchorage-independent growth state [16, 17], whether a tumor promoter actually induced transformation in HaCaT cells is unclear. Thus, we performed the anchorage-independent transformation assay with EGF in HaCaT cells. Results indicated that treatment with EGF induced colony formation on agar in a dose dependent manner (Figure 1A and B).

Effects of MAP Kinase Inhibitors on EGF-induced Signal Transduction

EGF mainly activates the ERK pathway [4,28,29], but also activates the JNK [30–33] and p38 pathway [6,34]. We confirmed that EGF induced ERK, JNK and p38 phosphorylation in HaCaT cells in a dose- (Figure 2A) and time-dependent (Figure 2B) manner. To study the relationship between MAP kinases and EGF-induced transformation, we utilized PD98059, SP600125 and SB203580, which are MEK, JNK and p38 inhibitors, respectively. We confirmed the effects of these inhibitors on signal transduction by immunoblotting (Figure 3). As expected, PD98059, SP600125 and SB203580 inhibited EGF-induced phosphorylation of ERK, c-Jun, a JNK substrate, and p38, respectively.

Physiological Effects of MAP Kinase Inhibitors in HaCaT Cells

Whereas ERK is activated by stimuli that promote growth and differentiation, p38 and JNK are activated strongly by environmental stress and inflammatory cytokines [20]. In contrast to ERK activation, the role of p38 and JNK activation in cell growth, proliferation and transformation has not been totally clarified. To delineate the role of ERK, JNK and p38 in EGF-induced transformation in a human cell model, we investigated the physiological effects of PD98059, SP600125 and SB203580 in HaCaT cells. First, we confirmed the effects of these inhibitors on cell growth under normal culture conditions (Figure 4A). PD98059 and SP600125 inhibited ERK and c-Jun phosphorylation and decreased cell growth. Even a 50% inhibition of phosphorylation by the inhibitors was associated with growth inhibition. However, the p38 inhibitor, SB203580, inhibited p38 phosphorylation (Figure 4B) by almost 50% at 2–10 μ M but slightly increased cell growth at 2 μ M. Next, we investigated the effects of MAP kinase inhibitors on EGF-dependent DNA synthesis by monitoring [3 H]-thymidine incorporation (Figure 5). HaCaT cells were cultured in 0.05% FBS/DMEM for 24 h and then stimulated by EGF. EGF stimulation increased [3 H]-thymidine incorporation in HaCaT cells, and PD98059 or SP600125, but not SB203580, inhibited the EGF-dependent [3 H]-thymidine incorporation.

Effects of MAP Kinase Inhibitors on EGF-Induced Transformation

Finally, we performed an anchorage-independent transformation assay with MAP kinase inhibitors (Figure 6). As expected, PD98059 or SP600125 inhibit EGF-induced colony-formation in a dose-dependent manner. However, p38 inhibition by SB203580 significantly enhanced EGF-induced colony-formation although it had no effect on EGF-dependent DNA synthesis.

DISCUSSION

Most of our present knowledge regarding the role of tumor related genes in neoplastic transformation originated from experimental animal model systems and cultured rodent cells. However, cell type differences obviously exist in the signaling pathways. For example, whereas Ras activation of Raf alone is sufficient to cause transformation of rodent fibroblasts, Ras requires Raf-independent pathways to cause transformation of a variety of epithelial cells [35–37]. In this study, we performed neoplastic transformation experiments with HaCaT cells, which are a spontaneously immortalized keratinocyte cell line developed from human adult keratinocytes [15], and utilized EGF as the tumor-promoter. The EGF receptor is overexpressed in tumors such as lung, colon, kidney and head and neck carcinomas [1,2], and approximately 30% of all human cancers express mutated and constitutively activated forms of Ras, an effector of the EGF receptor [38].

In mammalian cells, the ERK pathway is typically activated by growth factors and has a major role in cell proliferation and tumorigenesis because expression of constitutively active forms of components of this pathway can transform cells [39–41]. In the present study, pharmacologic inhibition of the ERK pathway decreased EGF-induced neoplastic transformation in HaCaT cells as expected. Indeed, elevated ERK activation has been associated with several human cancers [29,42,43]. Thus, the ERK pathway is clearly important for growth factor-induced neoplastic transformation in humans.

In contrast to ERK, the role of JNK and p38 activation in transformation has not been clearly delineated. In this study, we showed that the pharmacological inhibition of JNK by SP600125 suppressed cell growth and inhibited EGF-induced DNA synthesis and anchorage-independent growth in HaCaT cells. We, therefore, suggest that JNK activation might be required for neoplastic transformation induced by EGF in HaCaT cells. A role for JNK in oncogenic transformation and tumor development has been indicated in rodent cultured cells [44–46], and constitutive JNK activation induces cellular transformation in NIH3T3 cells [32,47]. In addition, the JNK pathway is required for EGF stimulated tumor growth in brain tumors, prostate carcinoma and lung carcinoma [30,31,33,48]. Moreover, the JNK substrate, c-Jun, is required for the transformation of mammalian fibroblasts by different oncogenes [49–51]. Taken together, JNK seems to be required for growth factor-induced transformation and tumor development in humans. On the other hand, JNK is also known as a mediator of apoptosis [52–54]. The distinct role of JNK1 and JNK2 isoforms might indicate a complicated regulation. Most studies suggest that JNK1 and JNK2 are functionally redundant [54–56]. However, in several cases, JNK1 preferentially mediates apoptosis [57–60]. Yang et al. [48] indicate that JNK2 plays a dominant role in the development of prostate carcinoma. JNK2 is preferentially required for EGF-induced transformation of human A549 lung carcinoma cells [31]. Furthermore, we have recently shown that JNK1 and JNK2 are critical in 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor suppression and promotion, respectively [61–63]. The role of JNK1 and 2 seems to depend on the kind of stimulation and type of cell. The JNK inhibitor, SP600125, inhibits both JNK1 and 2 activity [25], and therefore, we need further studies to investigate the role of each JNK isoform in EGF-induced neoplastic transformation with a genetic inhibition model.

Studies in rodent fibroblasts suggest that p38 acts as a negative regulator of Ras in cell transformation. For example, although transient expression of activated Ras was found to cause activation of p38, further activation of p38 blocked Ras-induced mitogenesis in NIH 3T3 cells [50] or reversed morphologic and growth transformation [64]. In addition, p38 can trigger cell cycle arrest at the G1/S cell cycle phase as part of a signaling pathway in late G1 mediated by Cdc42Hs and/or MEKK3 [20]. In HaCaT cells, p38 is activated by UVB, and then contributes to induction of apoptosis [65,66]. Importantly, Branchio et al. showed that the loss of p38

activation causes increased tumorigenesis *in vivo* [67]. p38 seems to mediate cell proliferation negatively. However, Chouinard et al. [68] reported that p38 protects normal human keratinocytes from cell death induced by UV irradiation, but has no protective effect on transformed HaCaT cells. Moreover, p38 is required for *c-fos* gene expression induced by UVB radiation [69,70]. In this study, we show that a p38 inhibitor enhanced cell growth and anchorage-independent transformation in HaCaT cells, and thus in this case, p38 seems to regulate cell growth and neoplastic transformation negatively. This result conflicts with the study in which mouse JB6 Cl41 cells were used [6], emphasizing the difficulties in applying data obtained from the mouse model to the human situation. Thus, the role of p38 in neoplastic transformation might be dependent on species, i.e. human vs. mouse. Another possibility is that p53 might be critical in explaining this difference. Because p53 phosphorylation is mediated by protein kinases, including p38 [71], the difference in p53 status (wild type in JB6 and mutated in HaCaT) also might explain the differential effect of the p38 inhibitor on EGF-induced transformation. Regardless, determining the role of p38 in cell transformation needs further study.

Whether the anchorage independent colonies will grow as tumors upon subcutaneous injection into nude mice is still to be determined but could provide further understanding of the transformation mechanism.

Finally, we showed that EGF treatment induced neoplastic transformation in the human immortalized keratinocyte cell line, HaCaT, suggesting the usefulness of HaCaT cells for studies of neoplastic transformation in humans. The transformation was suppressed by pharmacological inhibition of the ERK and JNK pathways, and enhanced by inhibition of p38 pathway, in HaCaT cells. Communication between different cellular signaling molecules is a well-established concept [72]. The EGF receptor is recognized as a convergence point for diverse signal transduction pathways [73]. Not only EGF, but also another growth factors, such as insulin-like growth factor, platelet-derived growth factor or fibroblast growth factor, are also possible critical factors for neoplastic transformation. p38 activation might be related to crosstalk between EGF and other growth factor related pathways although the detailed mechanism is not clear. Overall, our results provide further insight into the mechanisms of human cancer development.

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Figure 1.

Effect of EGF on neoplastic transformation in HaCaT cells. HaCaT cells (8000 cells) were exposed to EGF at various concentration (0 – 10 ng/ml). Cell colonies were scored after 14 days of incubation at 37 °C in a 5% CO₂ incubator. (A) Quantitation of total colonies from triplicate samples. Data indicate the mean $\pm$ S.D. ** = $p < 0.01$ compared to untreated control. (B) EGF (10 ng/ml) induces transformation in HaCaT cells.

Figure 2.

EGF-induced phosphorylation of ERK, JNK and p38 kinase in HaCaT cells. (A) After starving for 36 h in serum-free DMEM, HaCaT cells were treated with different concentrations of EGF (0 – 20 ng/ml) for 15 min. (B) After starving for 36 h in serum-free DMEM, HaCaT cells were incubated with EGF at 10 ng/ml for the indicated time. Proteins in total cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho ERK, anti-phospho JNK, anti-phospho p38 or anti-β-actin, as indicated.

Figure 3.

Effects of MAP kinase inhibitors on EGF-induced signal transduction. After starving for 36 h in serum-free DMEM, HaCaT cells were incubated with PD98059, a MEK inhibitor, SP600125, a JNK inhibitor, or SB203580, a p38 inhibitor, at different concentrations or with their vehicle, 0.1% DMSO as a control, for 1h. Then, HaCaT cells were treated with EGF at 10 ng/ml for 15 min. Proteins in total cell lysates were separated by SDS-PAGE and immunoblotted with anti-phospho ERK, anti-ERK, anti-phospho c-Jun, anti-β-actin, anti-phospho p38 or anti-p38, as indicated.

Figure 4.

Effects of MAP kinase inhibitors on growth of HaCaT cells. (A) HaCaT cells (1000 cells) were seeded in 96-well plates. After culturing for 24 h, medium was replaced with fresh DMEM containing 10% FBS. After culturing for 2–3 h, HaCaT cells were incubated with different concentrations of inhibitors as for Figure 3. After additional culturing for the indicated time, cell viability was measured by the MTS assay (Promega; Madison, WI). Data indicate mean $\pm$ S.D. ($n = 4$). * = $p < 0.05$ compared to control. (B) After culturing for 24 h in DMEM containing 10% FBS, HaCaT cells were incubated with inhibitor as for Figure 3 for 48 h. After adjusting protein amount, proteins in total cell lysates were separated by SDS-PAGE and

immunoblotted with anti-phospho ERK, anti-ERK, anti-phospho c-Jun, anti- β -actin, anti-phospho p38 or anti-p38, as indicated.

Figure 5.

Effects of MAP kinase inhibitors on EGF-dependent DNA synthesis in HaCaT cells. HaCaT cells (8000 cells) were seeded in 96-well plates. After culturing for 24 h, media were replaced with DMEM containing 0.05% FBS and cells were incubated for 24 h. HaCaT cells were incubated with inhibitors as for Figure 3 for 1 h. Then, HaCaT cells were treated with 10 ng/ml EGF. After culturing for 18 h, [³H]-Thymidine (0.5 μ Ci/well) was added to each well, and then cells were incubated for another 6 h. After harvesting of cells, the incorporation of the [³H]-thymidine was measured by liquid scintillation counting. Data indicate mean $\pm$ S.D. (n = 5). ** = $p < 0.01$ compared to vehicle.

Figure 6.

Effects of MAP kinase inhibitors on EGF-induced transformation in HaCaT cells. HaCaT cells (8000 cells) were exposed simultaneously to 10 ng/ml EGF and different concentrations of PD98059 (MEK inhibitor), SP600125 (JNK inhibitor), or SB203580 (p38 inhibitor) or their vehicle, 0.1% DMSO, as a control, in 0.33% agar BME containing 10% FBS over 0.5% agar BME containing 10% FBS. Cell colonies were scored after 14 days of incubation at 37 °C in a 5% CO₂ incubator. (A) PD98059 and SP600125, but not SB203580, inhibit EGF-induced transformation. (B) Quantitation of total colonies from triplicate samples. Data indicate the mean $\pm$ S.D. * = $p < 0.05$; ** = $p < 0.01$ compared to EGF alone.