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Activation of the extracellular signal-regulated kinases signaling pathway in squamous cell carcinoma of the skin

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SUMMARY **Activation of the extracellular signal-regulated kinase (ERK) pathway is involved in many human tumors. Little is known about the role of activated ERK1/2 in squamous cell carcinoma (SCC) of the skin. In this study, the expression and distribution of phosphorylated ERK (p-ERK) in normal human skin and SCC with different degrees of differentiation was examined by immunohistochemical analysis using formalin-fixed paraffin embedded sections. PD98059, a specific ERK pathway inhibitor, was used to evaluate the effect a blockade of ERK activation has on the proliferation of a cutaneous SCC cell line (DJM-1) in culture. In this study, p-ERK 1/2 positive staining was observed in all cases of SCC examined but rarely in the control specimens of normal skin. Moreover, the expression of p-ERK1/2 was significantly higher in poorly differentiated SCC in comparison to well-differentiated ones. Expression levels were positively associated with the degree of malignancy and proliferative activity of SCC. In contrast, inhibition of ERK pathway signaling markedly suppressed tumor cell proliferation. These results suggest that ERK1/2 signal pathways play an important role in the proliferation of SCC and that the inhibition of this signal pathway may be effective in the treatment of cutaneous SCC.**

Key Words: Extracellular signal-regulated kinases (ERK), squamous cell carcinoma (SCC), PD98059, proliferative activity, immunohistochemistry

Introduction

The mitogen-activated protein kinases (MAPKs) are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. In mammalian cells, there are three well-characterized subfamilies of MAPKs: the extracellular signal-regulated kinases (ERK), the c-Jun *N*-terminal kinases (JNK), and the p38 MAPK kinases (*1-3*). The ERKs are activated by most growth factors and have been shown to be a key regulator of both proliferation and differentiation in different cell types (*4*), while JNKs and p38 MAP kinase are activated by various forms of cellular stress and have predominantly been implicated in responses to cellular stress, inflammation, and/or apoptosis (*5*). There are three major MAP kinase pathways in human tissues, but that involving ERKs is most relevant to human cancer. A high level of p-ERK protein has frequently been observed in many human tumors (*6-10*). Furthermore, recent studies revealed that activation of ERKs plays a critical role in the proliferation of cancer cells (*11-14*). In contrast, the role of ERKs in cutaneous SCC was less clear. Therefore, the present study examined the expression of p-ERKs protein in normal skin and cutaneous SCC. PD98059 (a MEK/ERK inhibitor) was used as a tool to evaluate the effect of blockade of ERK activation on the proliferation of cutaneous SCC cell lines (DJM-1).

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Materials and Methods

Tissue samples

Surgically resected specimens used for this study included 5 portions of normal human skin obtained

from healthy patients undergoing plastic surgery; 10 had well-differentiated SCC and 10 had poorly-differentiated SCC. All tissue specimens were selected from the files of the Department of Dermatology & Plastic and Reconstructive Surgery, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University. Informed consent and institutional review board agreement were obtained. For each formalin-fixed and paraffin-embedded tissue block, several 4 μm sections were cut. One section was stained with H&E for histological examination, and the others were used for immunohistochemical staining.

Antibodies and reagents

Phospho-p44/42 MAP Kinase (Thr202/Tyr204) rabbit polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059 (a MEK/ERK inhibitor) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody and conjugate were included in the VECTASTAIN Elite universal ABC kit PK-6200 (Vector Laboratories, Burlingame, CA, USA).

Immunohistochemistry

Immunohistochemical staining was performed using the standard streptavidin-biotin-peroxidase complex method. Briefly, formalin-fixed paraffin sections 4 μm thick were deparaffinized and subjected to antigen retrieval by microwaving in 10 mM of citrate buffer (sodium citrate, pH 6.0) for 15 min. The sections were then treated with 0.3% hydrogen peroxide in methanol for 20 min at room temperature to block endogenous peroxidase. After they were washed in phosphate-buffered saline (PBS), unspecific binding sites were blocked with 5% normal horse serum at room temperature for 1 h. Excess serum was deleted from the sections. The tissues were then incubated with the primary antibody at 1:100 dilutions at 4°C overnight. Following washing with PBS, the sections were incubated with biotinylated horse-anti rabbit IgG at a dilution of 1:200 for 30 min at room temperature. The slides were rinsed and incubated with the avidin/biotin complex at room temperature for 60 min. Visualization of the peroxidase reaction was achieved with diaminobenzidine (DAB), followed by counterstaining with Giemsa.

A negative control slide for each tissue was incubated with non-immunized horse serum to replace the primary antibody.

Evaluation of immunohistochemical staining

Only nuclear staining was considered positive for p-ERK. The extent of immunoreactivity was evaluated in a semiquantitative manner using the following scale: Grade 1: < 5% of cells p-ERK positive; Grade 2: 5-25% of cells p-ERK positive; Grade 3: 26-50% of cells p-ERK positive; Grade 4: > 50% of cells p-ERK positive. Sections were examined in a double-blind manner to reduce bias and ensure consistency of examination.

Cell line and culture conditions

The human cutaneous squamous carcinoma cell line DJM-1 (*15*) was used in this study. The cells were routinely cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and antibiotics (penicillin, 100 U/mL and streptomycin, 100 mg/mL) at 37°C in a 5% CO_2 incubator. Cells were trypsinized and subcultured when they were approaching confluency.

Effect of PD98059 on cell proliferation

Confluent cells were harvested with an EDTA trypsin solution and re-suspended to appropriate concentrations in MEM medium containing 10% fetal bovine serum. After 1×10^4 cells/1mL growth medium was seeded in each well of a 24-well culture plate, cells were incubated 24 h to allow for attachment. Prior to addition of inhibitors, cells were cultured in serum-free MEM for 24 h to induce a quiescent state. Cells were then incubated for 24, 48 and 72 h in serum-free MEM containing either 5 μM, 10 μM, 20 μM, or 30 μM PD98059 (dissolved in dimethylsulfoxide (DMSO); final concentration in medium < 0.1%). In addition, cells incubated with serum-free MEM with 0.1% DMSO as a control. All experiments were performed in triplicate. The attached cell numbers were determined using a Coulter counter (Beckman Coulter, Fullerton, CA, USA).

Statistical analysis

Data are expressed as mean ± standard deviation. All experiments were performed in triplicate. Significant differences among the groups were determined using the Mann-Whitney *U*-test. A value of $p < 0.05$ was considered significant.

Results

Expression of p-ERK protein in normal control skin and normal skin adjacent to a tumor

In the control specimens of normal skin, no positive staining was seen in the epidermis and hair follicles; p-ERK immunoreactivity was observed in luminal surface of the acrosyringium and in the luminal surface and the nuclei of luminal cells in intra-dermal portions

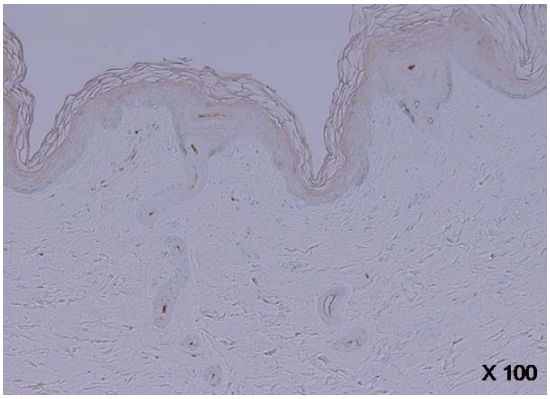

Figure 1. Expression of p-ERK protein in normal control skin. Expression was seen in the luminal surface of the acrosyringium and in the luminal surface and the nuclei of luminal cells in intradermal portions of the eccrine sweat ducts. A few vascular endothelial cells with weak nuclear staining were also seen. No signal was seen in the epidermis.

Table 1. Summary of p-ERK expression in 20 cases of SCC

Histological typing	Total	p-ERK expression			
		Grade 1 (< 5 %)*	Grade 2 (5-25%)*	Grade 3 (26-50%)*	Grade 4 (> 50%)*
Well-differentiated	10	2	6	2	
Poorly differentiated	10			3	7

P-ERK, phosphorylated extracellular signal-related kinase; SCC, squamous cell carcinoma. *Percentage of p-ERK positive cells of tumor cells in the specimen.

of the eccrine sweat ducts. Weak nuclear staining was occasionally found in some vascular endothelial cells (Figure 1). A similar expression pattern was obviously strengthened in normal skin adjacent to a tumor.

Expression of p-ERK protein in cutaneous squamous cell carcinoma

Results are summarized in Table 1 and illustrated in Figure 2. Nuclear staining of p-ERK protein was detected in all SCC specimens investigated; there was an obvious difference in the expression levels between poorly differentiated SCCs and well-differentiated SCCs. Immunohistochemical analysis showed that expression of p-ERK significantly increased in poorly differentiated SCCs in comparison to well-differentiated SCCs. In terms of the percentage of positive tumor cells, two cases of well-differentiated SCCs were grade 1, six were grade 2 and two were grade-3. However, three cases of poorly differentiated SCCs were grade 3 and seven were grade 4. Findings demonstrated that p-ERK expression was closely correlated with the degree of tumor cell differentiation. Even in well-differentiated SCCs, only the peripheral cells of the tumor nests were p-ERK-positive, but the central keratin pearls showed a negative immunoreaction.

Effect of PD98059 on cell proliferation

Results are shown in Figure 3. Human cutaneous squamous carcinoma cells, DJM-1 cells, were

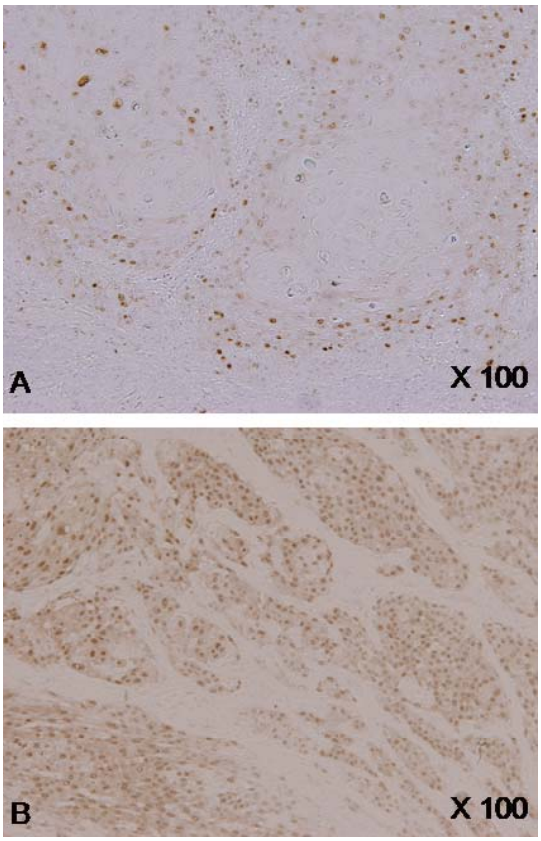

Figure 2. Expression of p-ERK protein in cutaneous squamous cell carcinoma. (A) In well-differentiated SCC, nuclear positive staining was noted in the less differentiated cells in the periphery of tumor cell nests. (B) In poorly-differentiated SCC, strong nuclear staining was present in the majority of tumor cells.

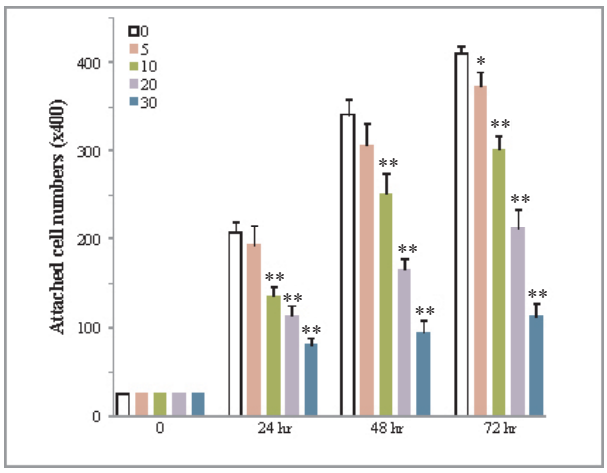

Figure 3. Effect of PB98059 on DJM-1 cell proliferation. Cells were plated in 24-well culture plates at a density of 1×10^4 cells/well in serum-supplemented medium. After a 24 h-attachment period, the cells were grown under serum-free conditions for 24 h. Cells were then incubated in serum-free MEM with different concentrations of PD98059 (0, 5, 10, 20, and 30 μM). The numbers of attached cells were counted at 24, 48, and 72 h after PD98059 treatment using a Coulter counter. Data are expressed as the mean ± standard deviation of three independent experiments. $*P < 0.05$; $**P < 0.01$ when compared to 0 (DMSO also).

incubated with PD98059 for different periods of time at concentrations ranging from 0-30 μM and the cell number was determined with a Coulter counter (Beckman-Coulter). PD98059 was shown to inhibit the proliferation of DJM-1 cells in a dose- and time-

dependent manner. Results showed that SCC cells were extremely sensitive to the growth-inhibiting effects of PD98059, which at a concentration of 30 μM almost completely suppressed DJM-1 cell proliferation.

Discussion

In the present study, nuclear staining of p-ERK protein was found in all SCC samples investigated. p-ERK expression was significantly higher in poorly-differentiated SCC than in well-differentiated SCC. Even in well-differentiated SCCs, expression was also limited to the less-differentiated area of the tumor, and staining was rarely detected in large keratinized cells at the centre of cell nests or horny pearls. These results revealed that the expression levels of p-ERK increased in accordance with decreasing grades of histological differentiation, suggesting that up-regulation of p-ERK expression reflects a high degree of malignancy and proliferative activity of SCCs.

Among MAPK pathways, the ERK pathway, known to be responsible for unregulated cell proliferation, is thus far one of the best characterized and is closely related to human cancer. High levels of phosphorylated ERKs have been reported in various types of human carcinoma cells (*6-10*). The elevated expression of p-ERK observed in SCCs in this study is consistent with the results of previous studies indicating that increased expression of p-ERK was correlated with more aggressive tumor behavior and higher proliferative activity (*8,11,13,14,16*).

In order to further confirm the functional role of activated ERK in the proliferation in SCCs, experiments were performed *in vitro* using MEK/ERK inhibitor PD98059 to treat DJM-1 in culture. DJM-1 was almost completely suppressed by PD98059 at a concentration of 30 μM. These results corroborate previous experimental studies that suggest p-ERK plays a critical role in the proliferation of malignant tumors (*11-14*).

Multiple factors are associated with the constitutive activation of the ERK pathway, including MEK-dependent and independent mechanisms (*17,18*). In cutaneous SCC cells, the MEK/ERK pathway inhibitor PD98059 completely inhibited cell proliferation, strongly suggesting that MEK-dependent mechanisms are involved. These data suggest that the MEK/ERK pathway is important for cutaneous SCC cell proliferation.

In summary, increased p-ERK is expressed in human cutaneous SCC and is related to proliferative activity. Inhibition of the MEK/ERK signal pathway with PD98059 completely eliminated SCC cell proliferation *in vitro*. Taken together, these results indicate that the MEK/ERK signal pathway may be an important potential therapeutic target in cutaneous SCC.

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