

The Air Liquid-interface, a Skin Microenvironment, Promotes Growth of Melanoma Cells, but not Their Apoptosis and Invasion, through Activation of Mitogen-activated Protein Kinase

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Received November 17, 2009; accepted December 15, 2009; published online February 4, 2010

The air-liquid interface (ALI) is a common microenvironment of the skin, but it is unknown whether the ALI affects melanoma cell behaviors. Using a collagen gel invasion assay, immunohistochemistry, and Western blots, here we show that melanoma cell proliferation in cultures with an ALI is higher than melanoma cell proliferation in submerged cultures. Bromodeoxyuridine (BrdU) uptake, an indicator of cell proliferation, of melanoma cells at the ALI was about 3 times that of submerged cells, while ALI and submerged melanoma cells had similar levels of single-stranded DNA (a marker of apoptosis). The ALI enhanced the expression of Raf-1, MEK-1 and pERK-1/2 components of the mitogen-activated protein kinase (MAPK) cascade, in cells more than the submerged condition did. The increases in BrdU uptake and pERK-1/2 expression promoted by ALI was abolished by the MEK inhibitor, PD-98059. ALI-treated and submerged melanoma cells did not infiltrate into the collagen gel, and they showed no significant difference in the expression of the invasion- and motility-related molecules, matrix metalloproteinase-1 and -9, laminin 5, and filamin A. Our data indicate that the ALI, a skin microenvironment, accelerates the growth, but not the apoptosis or invasion, of melanoma cells through MAPK activation.

Key words: air-liquid interface, melanoma, proliferation, mitogen-activated protein kinase, collagen gel invasion assay

I. Introduction

Skin cells that are exposed to atmospheric air are situated at an air-liquid interface (ALI) [7]. Thus, the ALI is a common microenvironment of the skin. For example, the ALI promotes the growth and differentiation of normal kera-

tinocytes [22]. Moreover, the ALI accelerates the invasive growth of cutaneous squamous cell carcinomas relative to invasive growth in the submerged condition [11]. ALI is thus considered to be a critical factor for both normal and neoplastic growth of skin cells.

Melanoma, a critical skin neoplasm currently responsible for about 80% of all skin-cancer-related deaths [15], arises from epidermal melanocytes or their precursor cells [3]. Thus, epidermal microenvironments, including the ALI, may be crucial for the initiation and progression of melanoma. Furthermore, the prognosis of melanoma with ulcer-

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ation is worse than that without ulceration [8]. Skin ulcer causes cutaneous cell types under the epidermis to situate at an ALI by exposing them to atmospheric air, and this ulcer-induced ALI may be related to the growth and invasion of more aggressive melanoma. However, it is unclear whether the ALI skin microenvironment directly affects the biological behavior of melanoma cells.

To address this interesting issue, we studied the proliferation and invasion of melanoma cells in a collagen gel invasion assay with or without an ALI. Growth-, invasion-, and motility-related molecules such as mitogen-activated protein kinase (MAPK) cascade proteins (Raf-1, MEK-1, and pERK-1/2), matrix metalloproteinase-1 and -9 (MMP-1 and -9), laminin 5, and filamin A [4, 12, 18, 26] were analyzed by immunohistochemistry and Western blot.

II. Materials and Methods

Cell lines

All procedures involving human materials were performed in accordance with the regulations laid down by the ethical guidelines of Saga University. We used two melanoma cell lines, KHm-1 (a kind gift from Prof. T. Kanzaki, Kagoshima University Graduate School of Medicine, Kagoshima, Japan) and HMY-1 (JCRB1018, Health Science Research Resources Bank, Osaka, Japan). KHm-1 [14] was established from a primary lesion of the back, and HMY-1 [16] was established from a metastasized lymph node lesion. The cell lines were maintained in the complete medium: minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 50 $\mu\text{g}/\text{ml}$ gentamicin.

Collagen gel invasion assay system

The collagen gel invasion assay system was organized as previously described [24]. Briefly, 2 ml type I collagen gel solution (Nitta Gelatin, Osaka, Japan) was poured into a 30-mm dish with a nitrocellulose bottom (Millicell-CM, Millipore, Bedford, MA) and incubated at 37°C for 30 min to solidify the gel. This inner dish was placed into a 90-mm outer dish, and the complete medium was added to both dishes. Then, 1×10^6 melanoma cells were spread onto the gel; the cells grew to confluence within 2 days. After 3 days in culture, the media of the inner and outer dishes were removed, and 10 ml of fresh complete medium was added to only the outer dish to create an ALI. The cells were exposed to humidified air supplemented with 5% CO_2 at 37°C. In this way, the cells were situated at ALI. In this system, the cells were kept moist and fed by culture medium that moved by capillary action from the outer dish, through acellular collagen gel layer, and into cellular layer. As a control, the cells on the gel were covered with complete medium and cultured in a submerged condition without an ALI. Figure 1 illustrates the experimental culture system.

Morphology and morphometric analysis of melanoma cell invasion

Collagen gel cultures containing the melanoma cells

were fixed with 4% formalin, embedded in paraffin, thin-sectioned vertically, deparaffinized, and stained with hematoxylin and eosin (H&E). Cellular stratification and downward growth of the cells into the gel were analyzed on the H&E staining sections by light microscopy, as described previously [24].

Immunohistochemistry

To characterize the growth of melanoma cells, we examined the expression of molecules in the MAPK cascade (Raf-1, MEK-1, and pERK-1/2) important for cell growth signaling [6, 20], using mouse monoclonal anti-Raf-1, anti-MEK-1, and anti-pERK-1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Raf-1 and MEK-1 antibodies were not phospho-specific, while pERK-1/2 antibody was phospho-specific. Immunohistochemistry was carried out on deparaffinized sections by an avidin–biotin complex immunoperoxidase method, as described previously [24].

Growth and apoptosis

We assayed cell proliferation and apoptosis on the seventh day in culture with or without ALI. To assess cell proliferation, cell cultures were incubated with 3

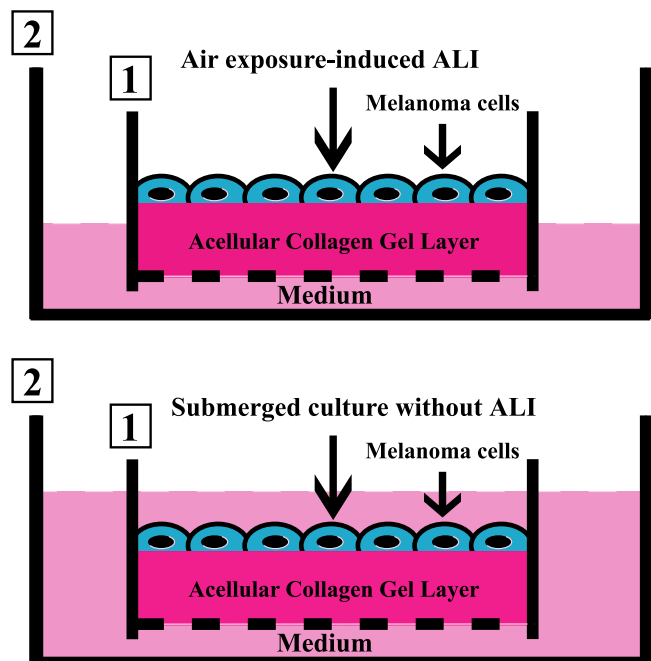


Fig. 1. Collagen gel invasion assay system with (upper panel) or without ALI (lower panel). Upper panel, Melanoma cells are seeded on the acellular gel layer in inner dish (1). The inner dish (1) is put in outer dish (2) with culture medium. In this way, the cells on the gel layer are localized under air exposure-induced ALI. The cells are kept in moist and fed by culture medium that percolates by capillary action from the medium-containing outer dish. Lower panel indicates submerged culture without ALI (control). In these systems, the invasion of melanoma cell into collagen gel is analyzed by the method described in Materials and Methods.

$\mu\text{g/ml}$ bromodeoxyuridine (BrdU, Cell Proliferation Kit, Amersham, Arlington Heights, IL) for 12 hr, and BrdU incorporation was detected using immunohistochemistry as described elsewhere [24]. Apoptotic cells were identified by the presence of single-stranded DNA (ssDNA), which was detected by immunohistochemistry using anti-ssDNA antibody (Dako Japan), as described previously [12]. For each assay (BrdU or ssDNA), 1000 cells were counted, and the percentage of positive cells was calculated.

Western blot

Melanoma cells, cultured for 7 days, were scraped from six inner dishes. They were homogenized in 10 mM sodium orthovanadate (Na_3VO_4) supplemented with protease inhibitors (Protease Inhibitor Cocktail Set, Boehringer-Mannheim, Tokyo, Japan). The lysates were centrifuged, and the supernatant was lyophilized, as described previously [24]. The lyophilized material was dissolved in 0.5 ml distilled water. Twenty micrograms aliquots of each protein sample were immunoprecipitated with Raf-1, MEK-1, pERK-1/2, matrix metalloproteinase-1 (MMP-1), MMP-9, laminin 5, or filamin A antibody (noted above) and protein A/G-agarose (Boehringer-Mannheim) in an EBC buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, and 10 mM Na_3VO_4 at 4°C for 2 hr. After centrifugation, each pellet was washed three times with ice-cold EBC buffer. After the final wash, each pellet collected by centrifugation was resuspended in 20 ml of electrophoresis sample buffer. Fifteen microliters of the sample was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane sheet. The sheet was incubated with each of the antibodies listed above. The antigen on the membrane was visualized by the standard method. The bands were quantified by densitometry. The protein expression levels for cultures with an ALI were calculated as a percentage of the densitometry values derived from cultures in the control submerged condition.

Effects of the MEK inhibitor PD-98059 on melanoma cell behavior

PD-98059, a MEK inhibitor that leads to the inactivation of ERK-1/2 (MAPK) [19], (Biomol International, LP) was added at a final concentration of $50 \mu\text{M}$ to melanoma cells cultured with an ALI at the initiation of the culture. The morphology of melanoma cells, their BrdU uptake, and the pERK-1/2 expression in such cells were analyzed on the seventh day of culture using the methods described above.

Statistical analysis

The data obtained from five to seven independent trials of each quantitative assay were analyzed by one way ANOVA. Values represented means \pm SD. A p -value < 0.05 was considered significant.

III. Results

ALI promotes proliferation of melanoma cells, but not their apoptosis or invasion

The ALI cultures had accelerated stratification of melanoma cells relative to the submerged cultures without an ALI (Fig. 2). BrdU uptake in melanoma cells with an ALI

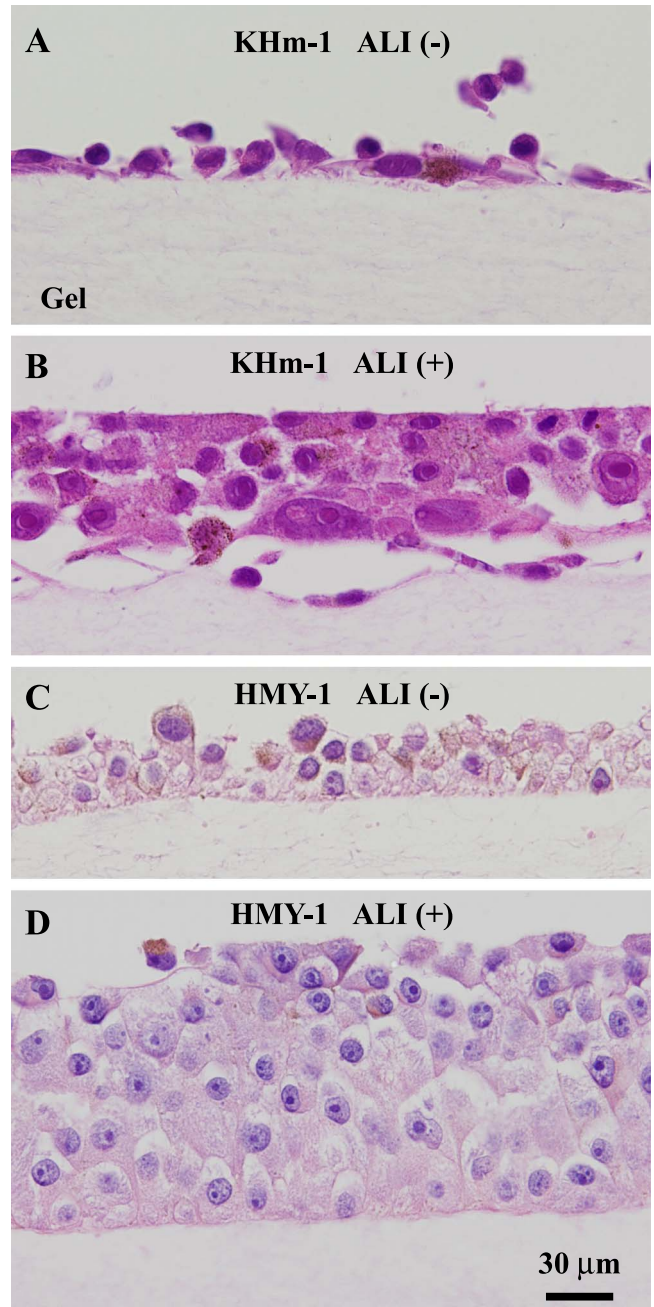


Fig. 2. Histology of two melanoma cell types of KHm-1 (A and B) and HMY-1 (C and D) with (B and D) or without ALI (A and C). ALI promotes the stratification of KHm-1 and HMY-1 melanoma cell types more greatly than submerged condition without ALI. In all conditions, the infiltration of these melanoma cell types into the gel was not observed. Brown pigments indicate melanin pigments.

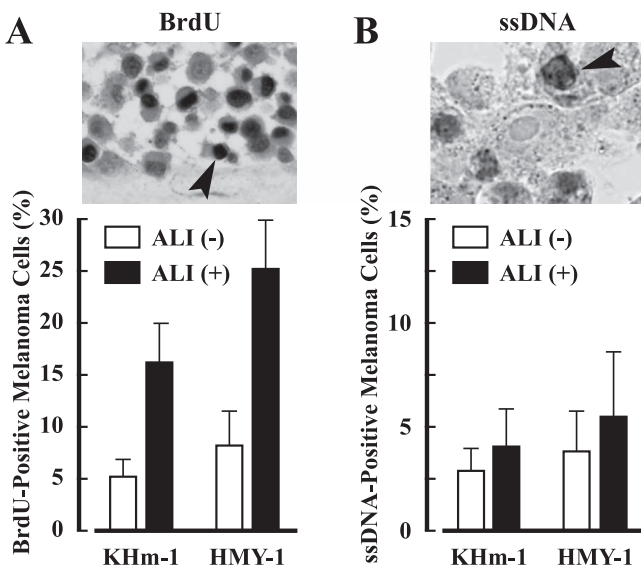


Fig. 3. Growth (A) and apoptosis (B) of melanoma cells with or without ALI, using immunohistochemistry of BrdU (growth marker) and ssDNA (apoptosis marker). BrdU (upper panel in A) and ssDNA (upper panel in B) are clearly detected in the nuclei of KHm-1 melanoma cells in black (arrowhead). The BrdU uptake of melanoma cells with ALI was about 3 times that of the cells without ALI ($p < 0.001$), while melanoma cells with or without ALI showed no significant change in the apoptotic rate ($p > 0.05$).

was about 3 times that of cells without an ALI (Fig. 3A), while melanoma cells cultured with or without an ALI showed no significant difference in the apoptotic rate (Fig. 2B). Melanoma cells cultured with or without ALI did not invade the collagen gel (Fig. 2). These results suggest that ALI promotes the proliferation but not the apoptosis or invasion of melanoma cells.

ALI promotes the expression of MAPK cascade proteins but not the expression of invasion- and motility-related proteins in melanoma cells

The expression of Raf-1, MEK-1 and p-ERK-1/2 was higher in melanoma cells cultured with an ALI than in those cultured in a submerged condition without an ALI (Fig. 4). Melanoma cells with or without an ALI showed no significant difference in the expression of the following invasion- and motility-related molecules: MMP-1, MMP-9, laminin 5 and filamin A (Fig. 5). These results suggest that the ALI promotes the hyperexpression of the MAPK pathway proteins (along with the growth of melanoma cells) and that the ALI does not promote the expression of invasion or motility proteins in melanoma cells.

Effects of MEK inhibitor (PD-98059) on melanoma cell behavior

The MEK inhibitor PD-98059 inhibited the stratification of melanoma cells cultured with an ALI (Fig. 6A, 6B) and induced their spindle-shaped morphology (Fig. 6B). PD-98059 also abolished the ALI-associated uptake of BrdU

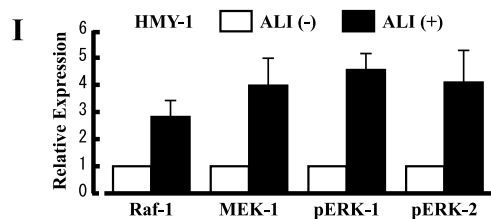
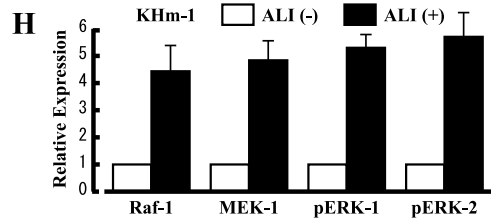
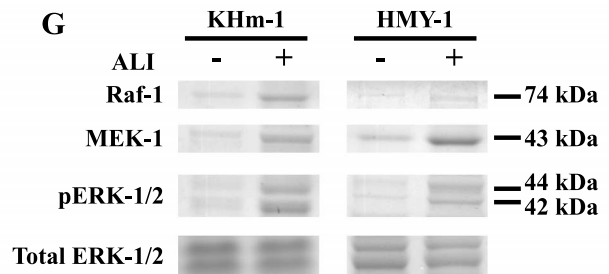
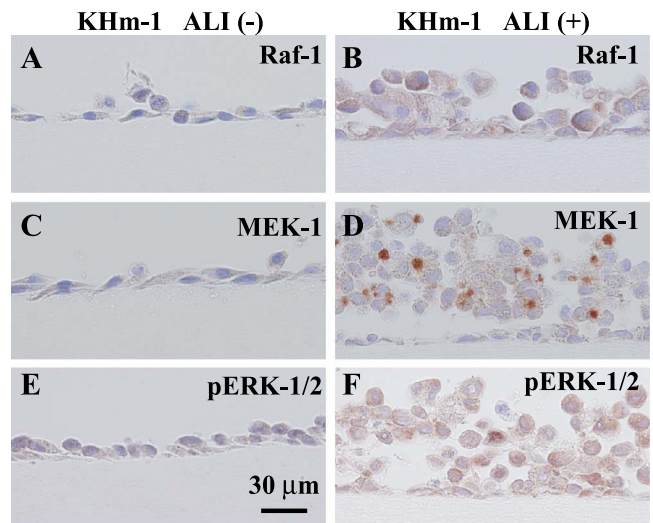


Fig. 4. Expression of MAPK pathway (Raf-1, MEK-1 and ERK-1/2) of melanoma cells with or without ALI. In immunohistochemistry (A-F), KHm-1 melanoma cells with ALI (B, D and F) express Raf-1 (A and B), MEK-1 (C and D) and ERK-1/2 (E and F) more extensively than those without ALI (A, C and D). Western blotting (G) confirmed the immunohistochemical results. Analyses by densitometry (H and I) show that ALI significantly enhances Raf-1, MEK-1 and pERK-1/2 expression of KHm-1 (H) and HMY-1 (I) melanoma cells (all values, $p < 0.001$). Total ERK-1/2 expression of melanoma cells with or without ALI had no significant change.

(Fig. 6C) and the expression of pERK-1/2 in melanoma cells (Fig. 6D).

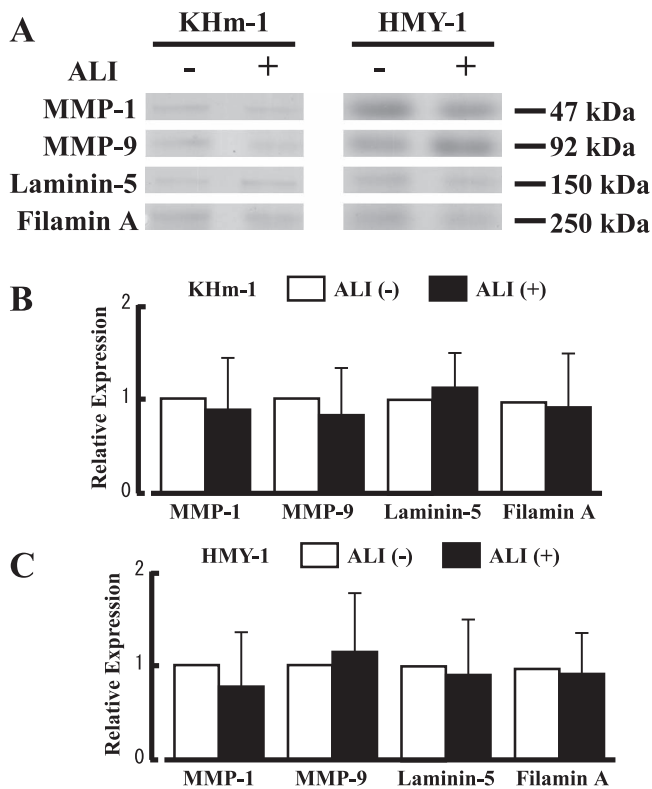


Fig. 5. Expression of MMP-1, MMP-9, laminin 5 and filamin A of KHm-1 and HMY-1 melanoma cell types with or without ALI by Western blotting (A) and analyses by densitometry (B and C). MMP-1, MMP-9, laminin 5 and filamin A are weakly detected in KHm-1 and HMY-1 melanoma cells types with or without ALI. Analyses by densitometry show that ALI does not significantly affect these molecule expression of KHm-1 (B) and HMY-1 melanoma cell types (C) (all values, $p > 0.05$).

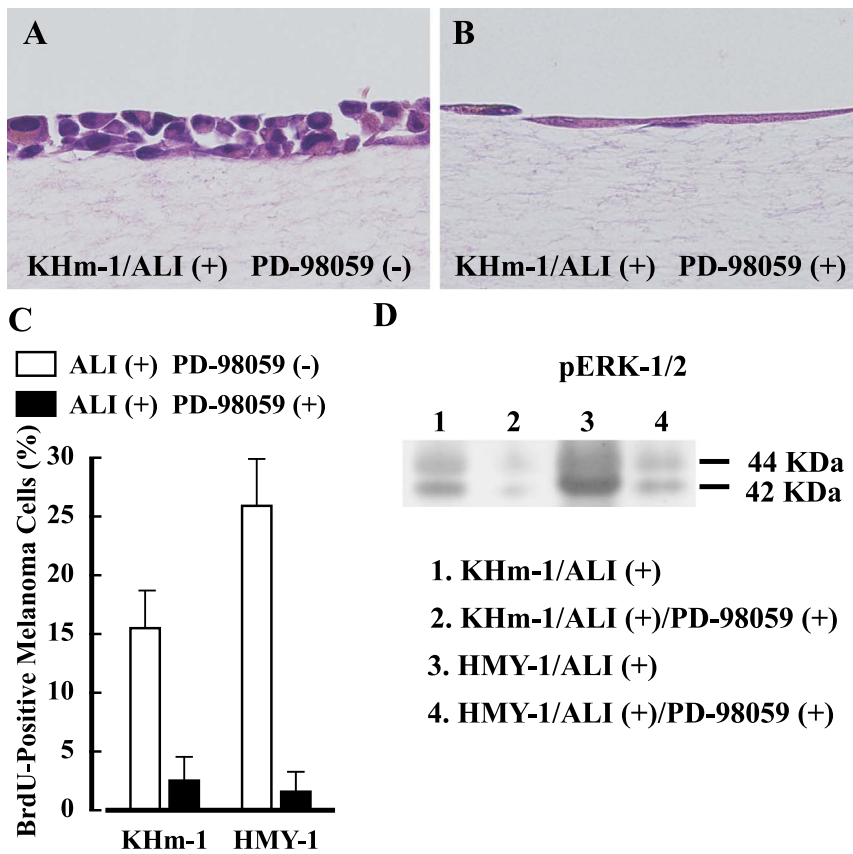


Fig. 6. Effects of the MEK inhibitor PD-98059 on the morphology, BrdU uptake and MAPK (ERK-1/2) expression of melanoma cells with ALI. ALI-treated KHm-1 melanoma cells without PD-98059 (A) undergo cellular stratification on the gel. In contrast, the melanoma cells with PD-98059 show no stratification and they become spindle-shaped (B). In ALI-treated KHm-1 and HMY-1 melanoma cell types, PD-98059 significantly inhibits both BrdU uptake (C, $p < 0.0001$) and pERK-1/2 expression (D) of the cell types.

IV. Discussion

Here we have shown for the first time that the ALI, a skin microenvironment, promotes the growth of melanoma cells, but not their apoptosis or invasion, more extensively than the submerged condition without an ALI does. The ALI also enhances the expression of components of the growth-related MAPK signaling pathway (Raf-1/MEK-1/ERK-1, 2) in melanoma cells, but not the expression of the invasion-related molecules MMP-1, MMP-9, laminin 5, and filamin A. These results suggest that the ALI is a critical factor for melanoma cell proliferation. Thus, the ALI microenvironment of the skin should be taken into account when considering the etiology and treatment of malignant melanoma.

As described in this study, ALI-promoted growth of melanoma cells is clearly inhibited by PD-98059, the MEK inhibitor that leads to the inactivation of ERK-1/2 (MAPK) [19]. Also, PD-98059 reduced the BrdU uptake and the expression of MAPK (pERK-1/2) in melanoma cells. This result suggests that the skin microenvironment ALI may cause melanoma cells to overgrow due to activation of MAPK. Our present study supports several reports [10, 17] that MAPK is closely involved in the activation of cell growth. However, the basic mechanisms of ALI-induced MAPK activation are unclear. In general, the ALI is thought to exert its effects by increasing the oxygen available to cells, which is important for cellular respiration [2, 21, 25]. Therefore, oxygen and oxidative stress molecules may be involved in ALI-promoted MAPK activation. In addition, BRAF mutation is closely associated with MAPK (ERK-1/2) activation of melanoma cells [23]. To clarify the mechanisms of ALI-stimulated signal transduction, further studies regarding the relationship between ALI-promoted MAPK activation and BRAF mutation are needed. Furthermore, the following possible mechanisms of ALI-promoted growth of melanoma cells may be considered: (1) diffusion of oxygen through cell membrane may take place more easily in the fluid-poor microenvironment of the ALI relative to the fluid-rich environment of the submerged condition without an ALI; (2) cell growth-related molecules may be more concentrated within the fluid-poor microenvironment of the ALI; and (3) the ALI may directly activate cell membrane microdomains (rafts) that regulate a variety of signal transduction molecules [5, 13]. To evaluate these possibilities in more detail, further studies are needed. In particular, gas molecules and signaling pathways important to cell growth, proliferation, invasion, and locomotion should be investigated in the context of ALI-cell membrane interactions. In addition, the MEK inhibitor PD-98059 clearly induces the melanoma cells to take on a spindle-shaped morphology. The significance of this morphological change is unclear at present. In general, melanoma cells often become pleomorphic in their metastatic sites [1]. It seems likely that MAPK inhibition may be involved in the mechanisms of the pleomorphism of melanoma cells.

In this study, ALI does not induce the invasion of melanoma cells into the collagen gel. Moreover, the ALI does

not promote the expression of MMP-1, MMP-9, laminin 5, or filamin A in melanoma cells, although melanoma cells cultured with or without an ALI express these molecules weakly. Our previous study [24] showed that the ALI promotes MMP-1 and filamin A expression in squamous cell carcinoma cells (HEp-2) of the larynx, enhancing their invasion into the gel of the collagen gel invasion assay system. Taken together, these results suggest that the effects of the ALI on metastasis and invasion may be cancer cell type dependent.

The prognosis of melanomas with ulceration is worse than that of melanomas without ulceration [8]. Skin ulcer seems to cause the cutaneous cell types of the dermis, those under the epidermis, to situate at the ALI by the ulcer-induced exposure of these cell types to atmospheric air. In general, normal and cancerous cells are supported in the interstitium by two major types of solid structures: (1) collagen fiber bundles and (2) proteoglycan filaments [9]. The interstitial fluid, which is derived by filtration and diffusion from capillaries, is entrapped mainly in the minute spaces among proteoglycan filaments. The proteoglycan filaments and the fluid entrapped within them (gel water) organizes a moist tissue gel in the interstitium, while intravascular space has rich fluid (free water) due to the presence of ample serum and the lack of a matrix meshwork [9]. In the moist gel, oxygen and nutrients are also supplied from capillaries. In this way, an ALI microenvironment seems to be created even in the interstitium of the dermis. Thus, the melanoma cell overgrowth that is activated by the ulcer-induced ALI microenvironment of the dermis may be relevant to the mechanisms of the worse prognosis for melanomas associated with ulceration [8].

In conclusion, we have shown for the first time that the ALI, a skin microenvironment, extensively promotes the proliferation of melanoma cells through MAPK activation, but not their apoptosis or invasion. This suggests that the ALI may be an important consideration in the etiology, progression, and treatment of melanoma.

V. Acknowledgements

We wish to thank Messrs. H. Ideguchi, F. Mutoh, S. Nakahara, and Mrs. M. Nishida for their excellent technical assistance. This work was supported in part by Grants-in-Aid from Japanese Ministry of Education, Culture, Sports, Science and Technology for Scientific Research Nos. 18591871 and 20592023 (to S. T.), and personal Grants from the Koike Hospital, Sasebo Chuo Hospital and Yamada Clinic (to S. T.).

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