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Original Article

Respective optimal calcium concentrations for proliferation on type I collagen fibrils in two keratinocyte line cells, HaCaT and FEPE1L-8

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ABSTRACT

Keratinocyte line cells HaCaT and FEPE1L-8 are used for skin model with type I collagen fibrils (gels). For this purpose, not only differentiation but also regulation of proliferation on type I collagen gels by exogenous calcium concentration is important. When exogenous calcium concentration is low, primary keratinocyte proliferation is repressed and eventually cells are induced to apoptosis on type I collagen gels. The apoptosis induced on type I collagen gels is suppressed by increasing calcium concentration in the medium. That is, higher exogenous calcium concentration is necessary for primary keratinocyte survival on type I collagen gels than for that on dish surface culture. Meanwhile much higher exogenous calcium causes cell differentiation and inhibition of proliferation. The optimal calcium concentrations for proliferation on type I collagen gels have not been clarified in keratinocyte line cells. HaCaT cells have a unique calcium sensitivity in comparison with primary keratinocytes, whereas FEPE1L-8 cells have a similar sensitivity to primary keratinocytes. In this study, we compared the effect of calcium concentrations on proliferation of HaCaT and FEPE1L-8 cells on type I collagen gels. On type I collagen gels, both line cells required higher calcium concentrations for proliferation than on dish surface. HaCaT cells proliferated better in a wider range of calcium concentrations than FEPE1L-8 cells.
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1. Introduction

Keratinocyte culture system is important but arduous. Boyce and Ham have developed an improved serum-free culture system for normal epidermal keratinocytes in 1983 [1]. The resulting optimized nutrient medium, MCDB 153, is sufficient for keratinocyte culturing without feeder layers, conditioned medium, or FBS. The calcium concentration in MCDB 153 medium is 30 μM. When calcium concentration is elevated to 1 mM in MCDB 153 medium, normal epidermal keratinocytes reaches stratification and terminal differentiation [1]. Several serum-free media have been optimized for epidermal keratinocytes, for example K110 type II (K110) including 30 μM calcium (Kyokuto Seiyaku Inc., Tokyo, Japan) [2,3] and KGM-Gold™ (Lonza Japan, Tokyo, Japan) [4].

HaCaT, which is a major line cell of keratinocyte, is spontaneously immortalized human keratinocyte line cells during changing procedures of culture conditions, temperature and exogenous

Abbreviations: DAG, diacylglycerol; DMEM, Dulbecco's Modified Eagle's Medium; DMEM (0), DMEM supplemented without fetal bovine serum; DMEM (10), DMEM supplemented with 10% fetal bovine serum; ECM, extracellular matrix; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; IP3, inositol trisphosphate; K110, K110 type II medium; MTT, 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide; PI, propidium iodide; PIP2, hydrolyze phosphatidylinositol bisphosphate; PI3K, phosphoinositide 3-OH-kinase; PKC, protein kinase C; WST-8, (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.

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calcium concentration [5]. HaCaT cells were initially established in a modified minimum essential medium containing a 4-fold concentration of amino acids and vitamins with 15% heat-inactivated fetal calf serum [5]. The cell culture systems have subsequently been developed to DMEM [6] or RPMI-1640 medium [7] supplemented with 5% or 10% FBS. Calcium concentrations of DMEM and RPMI-1640 media are 1.8 mM [8] and approximately 400 μM [9] respectively. Although normal keratinocytes produce differentiation markers when the calcium concentrations are increased to 1 mM [1,10], HaCaT cells in DMEM (10) containing 1.8 mM calcium continue to grow without differentiation [6]. However, further increase in calcium concentrations to 5 or 10 mM in DMEM inhibits DNA synthesis and HaCaT cell growth [11]. FEPE1L-8, which is another immortalized line cell of human keratinocytes, was generated by the transfection with the papillomavirus type 16 (HPV-16) transforming genes E6 and E7 from a human cervical carcinoma [12]. FEPE1L-8 is non-tumorigenic, but exhibit unlimited growth with limited differentiation potential. Like normal keratinocytes, FEPE1L-8 cells can be maintained in keratinocyte growth medium (KGM-Clonetics Corporation, San Diego, USA) [13] or K110 [3] as normal keratinocytes. Moreover, when calcium concentration is elevated to 1.8 mM in K110, FEPE1L-8 cells as well as normal keratinocytes produce involucrin, which is a protein substrate for transglutaminase in differentiated keratinocytes [14]. HaCaT cells are often used for skin model because of easy handling. FEPE1L-8 cells have also used in organotypic cultures [13].

Exogenous calcium concentration is important for cell culture. Increases in exogenous calcium concentrations trigger various intracellular signal transduction events, including those involved in cell survival, proliferation and differentiation [15–17]. Increasing in extracellular calcium concentrations inhibit proliferation of keratinocytes and induce differentiation via signal transduction [18]. For example, changes in calcium concentration stimulate phosphoinositide metabolism, providing critical second messengers for the differentiation [19]. Specifically, phospholipase C beta and gamma enzymes, PIP2 to IP3 and DAG, and the resulting in DAG and intracellular calcium concentrations activate PKC [20].

Also adhesion to ECM regulates signal pathways activation that determines cell fates. Type I collagen is a most ubiquitous ECM component of connective tissue and its fibrils form the framework of tissue. *In vitro* purified type I collagen molecules are reassembled into fibrils under physiological conditions that make up gels [3,21]. Type IV collagen and some kinds of laminins are major basement membrane components [22]. *In vivo* generally keratinocytes have contact with basement membrane not type I collagen [1,22]. Adhesion to ECM regulates survival or death signal pathways activation. For example, in keratinocytes on type I collagen gels, Akt activation is suppressed [3]. Akt is a serine/threonine kinase that plays critical regulatory roles in multiple cellular processes including survival [23]. When exogenous calcium concentration is low, on type I collagen gels primary human foreskin keratinocytes and FEPE1L-8 cells adhered to the substrate once but subsequently entered apoptosis without exhibiting signs of differentiation or Akt activation [3]. However, increased calcium concentrations suppressed the induction of apoptosis on type I collagen gels via MAPK activation. In agreement, human foreskin keratinocytes were previously shown to survive on type I collagen gels in the presence of 1.8 mM calcium, although Erk1/2 activation rather than Akt activation was reported [14]. Following specific integrin binding to specific ECM, signal pathways are activated [24]. Ligation of laminin 332 by integrin alpha 6 beta 4 activates PI3K signaling. This activation allows cells to adhere and spreading via integrin alpha 3 beta 1, on laminin 332 independent of RhoGTPase, a regulator of actin stress fibers [25]. In contrast, adhesion and spreading on type I and type IV collagen via alpha 2 beta 1 is Rho-dependent [25].

Because the optimal exogenous calcium concentration to proliferate on type I collagen gels have not been defined in HaCaT and FEPE1L-8 cells, in this study we examined proliferation of HaCaT and FEPE1L-8 cells on type I collagen gels under varied calcium concentrations.

2. Methods

2.1. Cell cultures

HaCaT cells were purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany) and they were maintained in DMEM (Sigma D6046; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Biowest Inc., Round Rock, TX, USA). After conditioning from DMEM (10) to serum-free keratinocyte medium, K110 (Kyokuto Seiyaku Inc., Tokyo, Japan), proliferating HaCaT cells were passaged using 0.05% trypsin at least five times (data not shown). FEPE1L-8 cells were kindly donated by Dr. W. G. Carter (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and they were maintained in K110.

2.2. Preparation of cell culture substrates

Acid-soluble collagen type I (ASC-1-100-20) (bovine skin) was obtained from Nippi, Inc. (Tokyo, Japan). Prior to cell culture, the plastic surfaces of the culture plates were treated with type I collagen. Molecular type I collagen (10 μg/mL in 1 mM HCl) solution was poured into the dishes and they were stored for 1 h at room temperature [3]. To assemble molecular type I collagen into fibrils (type I collagen gels), 1.0 mg/mL of neutralized collagen solutions were incubated in 96- and 6-well culture plates at 0.1 and 1 mL/well respectively, for 1 h at 37 °C in a CO_2 incubator [3,20]. Before cell culture, molecular type I collagen coated surfaces without gel form were blocked with 1% BSA in PBS (−) for 1 h at room temperature.

2.3. Antibodies

The anti-integrin antibodies, TS2/16 (anti-integrin beta 1), P1E6 (anti-integrin alpha 2), GoH3 (anti-integrin alpha 6), and Y9A2 (anti-integrin alpha 9), were purchased from Sigma Aldrich (St. Louis, MO, USA). The anti-integrin monoclonal antibodies, 3G8 (anti-integrin alpha 3) [26] and 8F1 (anti-integrin alpha 5) [27] were produced in the Sekiguchi Lab.

2.4. Flow cytometric analyses of integrin expression

HaCaT and FEPE1L-8 cells were grown in non-treated dish surface for 2 days, trypsinized at 37 °C for 5 min, washed twice in 0.5% BSA/PBS (−). 5.0×10^5 cells/mL cells were re-suspended in 0.1 mL of 1% BSA/PBS (−). After incubation in 1% BSA/PBS (−) for 30 min on ice, the cells were incubated with primary antibodies for 30 min on ice, washed three times in 0.5% BSA/PBS (−), and incubated with Alexa Fluor-488-labeled secondary antibody for another 30 min on ice. Cells were then washed with 0.5% BSA/PBS (−) and re-suspended in 0.5 mL of HBSS supplemented with 2% FBS and 1 mM HEPES. Immunofluorescent-stained cells were analyzed using a FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA) instrument, and data were analyzed using the FlowJo program (Tomy Digital Biology Co., Ltd., Tokyo, Japan).

2.5. Cell proliferation analysis

Prior to cell culture, the 96-well culture plates were pre-treated with type I collagen as described above. HaCaT cells (5.0×10^4 cells/

mL) or FEPE1L-8 cells (5.0×10^4 cells/mL) were cultured in 96-well culture plates with 0.1 mL of medium for indicated times. After washing out non-adherent cells, the relative numbers of viable cells were estimated using WST-8 modified tetrazolium salt cell proliferation kits (Cell Counting Kit-8; Dojin, Kumamoto, Japan) according to the manufacturer's protocol. Absorbance at a wavelength of 450 nm (OD450) was measured by using a microplate reader (SH-9000 Lab; Corona Electric Co., Ltd., Ibaraki, Japan). Three wells were examined for each condition.

2.6. Flow cytometric analysis of apoptotic cells

HaCaT and FEPE1L-8 cells were grown in 6-well plates on type I collagen gels or molecular type I collagen. Subsequently cells were suspended using collagenase treatment at 37 °C for 5 min, followed by trypsin treatment at 37 °C for 5 min. Cells were then re-suspended in 0.1 mL of 0.5% BSA/PBS (−), and cells were stained with MEBCYTO® Apoptosis Kit (Annexin V-FITC Kit; MBL, Nagoya, Japan) according to the protocol. Cells were finally analyzed using a MoFlo™ XDP cell sorter (Beckman Coulter, Inc., Brea, CA, USA) and data were analyzed using the Summit Software (Beckman Coulter, Inc.).

3. Results

3.1. Integrin expression of HaCaT and FEPE1L-8 cells

Integrins interact with specific ECM proteins. Integrin expression on cell surface may dictate cell adherence to ECM types [24]. Thus, expression levels of integrin subtypes were investigated in HaCaT and FEPE1L-8 cells using a flow cytometer (Table 1). In these experiments, the collagen receptor composition, integrin alpha 2 and the laminin receptors of integrins alpha 3 and alpha 6 were highly expressed in both line cells. In contrast, integrins alpha 5 and alpha 9 were very low. HaCaT cells have larger amounts of all the integrins examined than FEPE1L-8 cells. Expression level (mean values) of integrin alpha 6 was more than 2-fold higher in HaCaT cells than in FEPE1L-8 cells, and that of integrin alpha 3 was approximately 1.4-fold higher in HaCaT cells. HaCaT cells had larger amount of laminin receptors comparing to FEPE1L-8 cells.

3.2. Cell morphology and proliferation of HaCaT and FEPE1L-8 cells on molecular type I collagen and type I collagen gels

Molecular type I collagen and three-dimensional structure formed type I collagen gels give diverse effects on keratinocyte fates [3]. To compare the effects on cell morphology and proliferation of type I collagen structural forms, we cultured HaCaT and FEPE1L-8 cells on both forms of type I collagen. HaCaT cells, which were maintained in DMEM (10) had adhered and spread on both forms of type I collagen for the initial 2 h (Fig. 1A and B). 3-days later, on molecular type I collagen, cells had spread and reached confluence (Fig. 1C). Also spread on type I collagen gels (Fig. 1D) and kept to growing on both molecular type I collagen (Fig. 1I, solid line, filled circles) and type I collagen gels (Fig. 1I, dotted line, open circles). Living cell numbers on type I collagen gels was smaller than that on molecular type I collagen. After 3-day culture on both collagen forms, living cell numbers reached over 3 folds of those at 2 h after seeding (Fig. 1K, white bars).

FEPE1L-8 cells adhered and spread on both forms of collagen in the initial 2 h-culture (Fig. 1E and F). 3 days after seeding, on molecular collagen, the cells had spread well (Fig. 1G). In contrast, the cells showed limited spreading on type I collagen gels (Fig. 1H). FEPE1L-8 cells were kept to proliferate on molecular type I collagen (Fig. 1J, solid line, closed circles) and living cell numbers reached about 3-folds comparing for 2 h (Fig. 1K, white bar). Cells did not proliferate on type I collagen gels (Fig. 1J, dotted line, open circles, Fig. 1K, white bar) till 3 days. In both cells, the relative number of adherent cells on the type I collagen gels were lower than those on molecular type I collagen.

In early stage of apoptosis, phosphatidylserine on the plasma membrane surface translocate from inside layer to outer layer. Annexin V is a phospholipid-binding protein with high affinity for phosphatidylserine. When the phosphatidylserine translocation occurs, cell membrane loses its integrity and becomes leaky. In dead cells, both Annexin V and PI are stained in cells [28]. On molecular type I collagen 6 days after culture, Annexin V and PI positive cell ratios were low in both the cells. In HaCaT cells, the Annexin V positive ratio was 4.83% and the PI positive cell ratio was 5.36% (Table 2). Similarly, in FEPE1L-8 cells, Annexin V positive cell ratio was 0.260% and the PI positive cell ratio was 3.91% (Table 3). In contrast, HaCaT cells culture on type I collagen gels, the Annexin V positive cell ratio was 24.1% and the PI positive cell ratio was 18.5% (Table 2). Corresponding ratios of FEPE1L-8 cells on type I collagen gels were 30.1% and 23.8% respectively (Table 3). This means that the phosphatidylserine translocation occurred on-gel culture and weakened cell membrane rigidity in both cells.

3.3. Cell morphology and proliferation of HaCaT cells in K110

In order to avoid the effects of culture media, HaCaT cells were acclimatized to a K110 containing 30 μM calcium and maintained for at least 5 passages. FEPE1L-8 cells could not be maintained in DMEM (10). (data not shown).

Following acclimation to K110, HaCaT cells were cultured with 30 μM or 1.8 mM calcium concentration on molecular type I collagen and type I collagen gels. Cells adhered and spread on both substrates for several hours irrespective of calcium concentration (data not shown). After 2-day culture on molecular type I collagen in both 30 μM (Fig. 2A) and 1.8 mM (Fig. 2C) calcium concentration, the cells had spread, proliferated (Fig. 2E) independently of calcium concentration and reached confluence. Living cell numbers reached over 3 folds compared with those at 2 h (Fig. 2G).

However, in the presence of 30 μM calcium on type I collagen gels, HaCaT cell spreading was prevented (Fig. 2B) and proliferation was suppressed (Fig. 2F solid line, 2G). In contrast, in the presence of 1.8 mM calcium on type I collagen gels, cells had spread and formed aggregates (Fig. 2D, white arrow), proliferated and living cell numbers reached over 3-folds compared with that at 2 h (Fig. 2F dotted line, 2G).

In K110, after 6 days of culture of HaCaT cells on molecular type I collagen, the Annexin V positive ratio was 2.05% and the PI positive cell ratio was 3.67%. Moreover, these ratios were 20.7% and 13.6% respectively on type I collagen gels (Table 4). Annexin V and PI positive cell ratios of HaCaT cells on type I collagen gels were nearly equal between in DMEM (10) (Table 2) and in K110 with 30 μM calcium (Table 4). This phenomenon suggests that on type I

Table 1
Expression of integrins. Mean peak Fluorescence intensity units (Alexa488 FITC-H).

Integrin	HaCaT	FEPE1L-8	HaCaT/FEPE1L-8 (folds)
beta 1	23519	19940	1.18
alpha 2	10941	7644	1.43
alpha 3	14597	9788	1.49
alpha 5	893	460	1.94
alpha 6	16189	7527	2.15
alpha 9	291	164	1.77
IgG	285	359	0.79

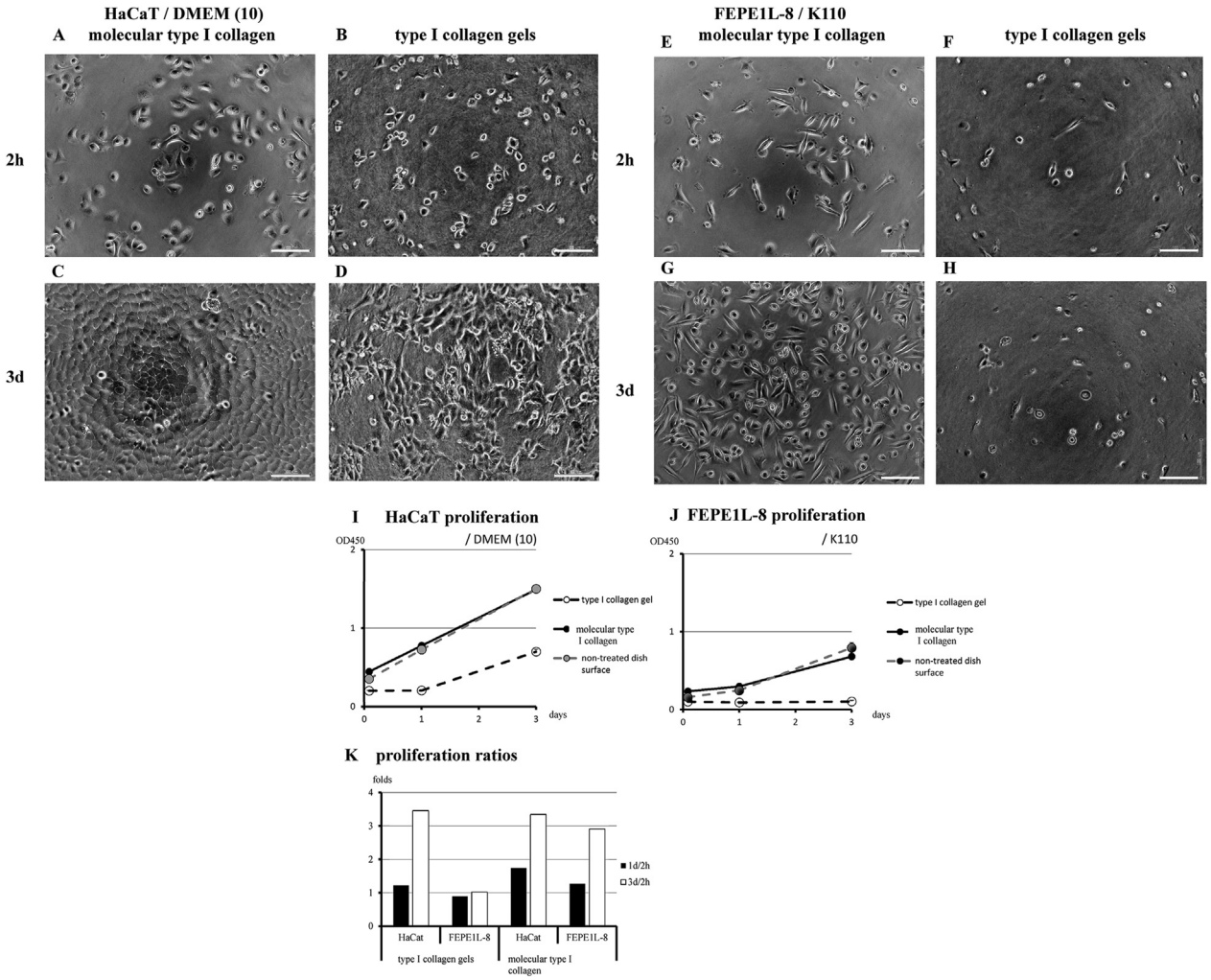

Fig. 1. Morphology and proliferation in keratinocyte line cells cultured on molecular type I collagen and type I collagen gels. HaCaT cells in DMEM (10) were cultured on molecular type I collagen (10 μg/mL) (A, C) or type I collagen gels (1 mg/mL) (B, D) for 2 h (A, B) or 3 days (C, D). FEPE1L-8 cells in K110 were cultured on molecular type I collagen (10 μg/mL) (E, G) or type I collagen gels (1 mg/mL) (F, H) for 2 h (E, F) or 3 days (G, H). White bars indicate 100 μm. Viable cell numbers of HaCaT (I) and FEPE1L-8 cells (J) were estimated on molecular type I collagen (solid line, filled circle), type I collagen gels (dotted line, open circle), or non-treated dish surfaces (dotted line, grey circle) at 2 h, 1day and 3days. Experiments were performed in triplicates and values are shown as means ± SD. Cell proliferation ratios are estimated by the averages of OD450 values at 1- or 3-day over those at 2 h for each culture conditions (K).

Table 2
HaCaT cells cultured for 6 days in DMEM (10) (Ca: 1.8 mM). Annexin V and PI positive ratio (%).

	Molecular type I collagen	Type I collagen gels
Annexin V positive rate (%)	4.83	24.1
PI positive rate (%)	5.36	18.5

collagen gel culture, certain apoptotic signals may be activated regardless of culture medial types. Interestingly, the HaCaT cell aggregates that formed in K110 with 1.8 mM calcium concentration were firmer than those formed in DMEM (10). Cell aggregates were not dispersed into single cells by collagenase and trypsin treatment described above. So, flow cytometric analysis was not performed.

Table 3
FEPE1L-8 cells cultured for 6 days in K110 (Ca: 30 μM). Annexin V and PI positive ratio (%).

	Molecular type I collagen	Type I collagen gels
Annexin V positive rate (%)	0.260	30.1
PI positive rate (%)	3.91	23.7

3.4. Comparison of calcium concentration dependency of proliferation in HaCaT and FEPE1L-8 cells on type I collagen gels

To form skin models, over several weeks of keratinocyte culture on type I collagen gels is necessary. Therefore, HaCaT and FEPE1L-8 cells were cultured on type I collagen gels in K110 at calcium concentrations, 30, 60, 90, 120, 150, 180, 450, 900 μM, 1.8 mM and 3.6 mM for a longer period. Numbers of viable cells at 2 and 7 days of culture were determined at each calcium concentration on type I collagen gels in HaCaT (Fig. 3A) and FEPE1L-8 cells (Fig. 3B). HaCaT cells proliferated very well during culture days 2 to day 7, when calcium concentrations were above 60 μM. Moreover, HaCaT cell numbers increased with calcium concentrations up to 1.8 mM. At a higher concentration, 3.6 mM calcium, cell number decreased slightly. Relative OD450 values at day 7 to those at day 2 were used for estimation of cell proliferation ratios at each calcium concentrations (Fig. 3C). HaCaT cell proliferation ratios reaching maximum at 900 μM calcium. After culture in 900 μM calcium for 7 days,

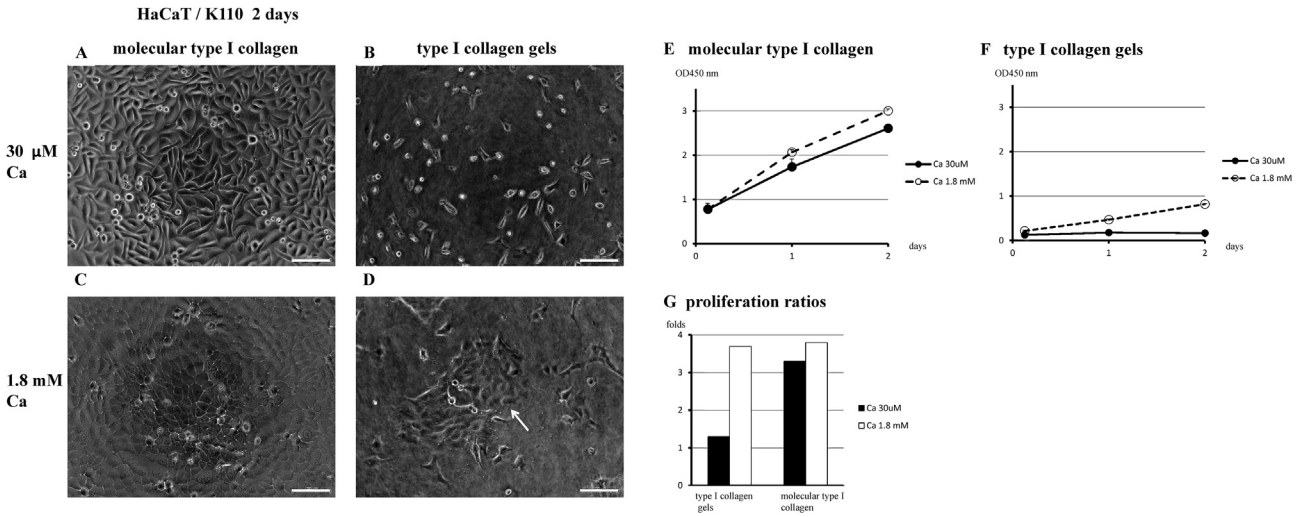

Fig. 2. Morphology and proliferation in HaCaT cells cultured on molecular type I collagen and type I collagen gels in K110. HaCaT cells in K110 were cultured for 2 days on molecular type I collagen (10 μg/mL) (A, C) or type I collagen gels (1 mg/mL) (B, D) with 30 μM (A, B) or 1.8 mM (C, D) calcium concentration. White arrow indicates cell aggregate (D). White bars indicate 100 μm. Viable cell numbers were estimated on molecular type I collagen (E) or type I collagen gels (F) with 30 μM (solid line, filled circle) or 1.8 mM (dotted line, open circle) calcium concentrations. All experiments were performed in triplicates and values are shown as means ± SD. Cell proliferation ratios are estimated by the averages of OD450 values at 2-day over those at 2 h for each culture conditions (G).

Table 4
HaCaT cells cultured for 6 days in K110 (Ca: 30 μM). Annexin V and PI positive ratio (%).

	Molecular type I collagen	Type I collagen gels
Annexin V positive rate (%)	2.05	20.7
PI positive rate (%)	3.67	13.6

living cell numbers of HaCaT reached over 6 times those on day 2 (Fig. 3C, solid line). In contrast, FEPE1L-8 cells did not proliferate in the presence of less than 90 μM calcium and cell proliferation reached a plateau at 180 μM calcium. The optimal calcium concentration for FEPE1L-8 cell proliferation was 180 μM, cell numbers increased by less than 3 times those on day 2 (Fig. 3C, dotted line). HaCaT cells grew better than FEPE1L-8 cells under any calcium concentrations. The data indicate that HaCaT cells proliferate under a wider range of calcium concentration than FEPE1L-8 cells. A schematic view of calcium ranges from proliferation starting point to reaching a plateau of the present keratinocyte cell lines are shown in Fig. 3D.

4. Discussion

It is known that keratinocyte proliferation and differentiation are affected by adhesion to ECM and exogenous calcium concentration. But the effects of type I collagen fibrils in combination with culture media on keratinocyte proliferation has not been well elucidated. The combination of these conditions is important for skin model formation. Thus, in the present study, we compared calcium concentration dependent proliferation on type I collagen fibrils in two different calcium sensitive keratinocyte line cells, HaCaT and FEPE1L-8. On type I collagen fibrils, apoptosis was induced in HaCaT and FEPE1L-8 cells like primary keratinocytes [3], though induction of apoptosis was slower than primary keratinocytes. In our previous report, we showed that apoptosis of keratinocytes on type I collagen fibrils in low calcium concentration is due to the lack of survival signals dependent Akt activation which is activated by integrin-mediated adhesion to laminin 332 or 511/521 [3]. We found that apoptosis of primary keratinocytes on type I collagen fibrils was rescued when cells were cultured in high calcium concentration [14]. By increasing calcium concentration in culture medium, laminin 332 deposition and Erk 1/2 activation occurred. Activation of Erk 1/2 may compensate for the loss of Akt activation, allowing keratinocyte survival on type I collagen fibrils. In wound healing site, laminin deposition on dermal collagen fibrils switches adhesion and signaling from collagen-dependent to laminin-dependent. Ligation of laminin 332 by integrin alpha 6 beta 4 activates phosphoinositide 3-OH-kinase signaling [25]. Interaction of integrins with laminin is critical for cell survival. Gu and coworkers reported that survival signals by the adhesion to laminin-10/11 (laminin 511/521) were activated mainly through the PI3-Kinase/Akt pathway [29]. Like primary keratinocytes, both HaCaT [30] and FEPE1L-8 [13] cells produce laminin, switching of adhesive substrates from collagen to laminin may as well play important roles for cell survival.

Despite the same origin of cells, HaCaT cells differ from FEPE1L-8 cells in multiple assessments. The present study shows that the expression of integrin was different. In HaCaT cells, especially integrin alpha 6 which is a laminin receptor was higher than that in FEPE1L-8 cells over twice (Table 1). Another different point was sensitivity for culture medium. HaCaT cells were more insensitive to the culture media compared with FEPE1L-8 cells. HaCaT cells can be maintained in either keratinocyte-dedicated [31] or non-dedicated medium [6,7] on non-treated plastic dish surface. In this paper, HaCaT cells could proliferate in both DMEM (10) and K110, but FEPE1L-8 cells could not be maintained in DMEM (10) on non-treated plastic dish surface. For calcium sensitivity in proliferation on molecular type I collagen and non-treated dish surface, there was no difference between HaCaT and FEPE1L-8 cells. Both cells proliferated to confluence at calcium concentrations of 30 μM to 3.6 mM with K110 (data not shown). But on type I collagen fibrils, a difference occurred between two cells. The present study showed that type I collagen fibrils made ranges of calcium concentrations narrow for the proliferation of both the cells. Calcium concentration range which appropriate for the proliferation of HaCaT cells is wider than that for FEPE1L-8 cells.

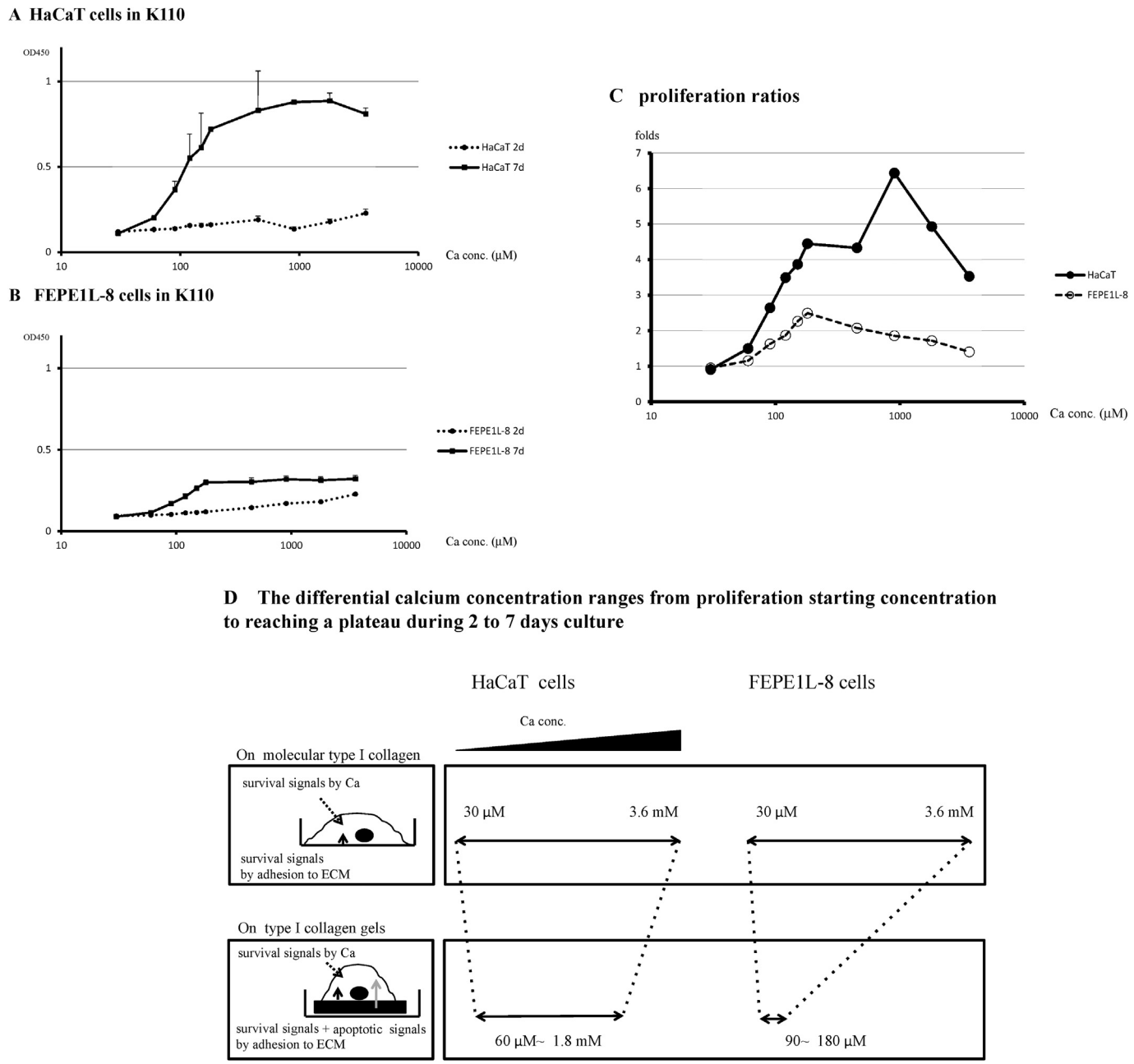

Fig. 3. Proliferation ratios of keratinocyte line cells cultured on type I collagen gels in K110 with various calcium concentrations. HaCaT (A) and FEPE1L-8 cells (B) in K110 were cultured on type I collagen gels with 30, 60, 90, 120, 150, 180, 450, 900 μM, 1.8 mM and 3.6 mM calcium concentrations for 2 days (dotted line) or 7 days (solid line), estimated living cell numbers using MTT assay. All experiments were performed in triplicates and values are shown as means ± SD. After 2 to 7 day-culture, proliferation ratios are estimated by average OD450 values at 7 days over those at 2 days in HaCaT (solid line) and FEPE1L-8 (dotted line) (C). Schematic views of exogenous calcium range from the beginning of cell proliferation to a plateau between 2 and 7 days were shown in D. Survival signals are activated by adhesion to both forms of type I collagen (solid black arrows) and exogenous calcium stimulus (dotted black arrows). In cells on molecular type I collagen, apoptosis induction did not occur. On type I collagen gels, apoptosis was induced in 20–30% both of HaCaT and FEPE1L-8 cells (Tables 3 and 4). On type I collagen gels, apoptotic signals are activated as well (grey arrow). Increasing exogenous calcium concentration reinforces activation of survival signals and cell proliferation to some extent (D).

HaCaT keratinocytes have been widely employed for the construction of skin model, reflecting the easier propagation as well as their near-normal phenotypes. However, HaCaT cells differ from not only primary keratinocytes but also keratinocyte line cells FEPE1L-8, particularly in their insensitivity to calcium and culture media contents. Further studies of differential properties between HaCaT cells and other keratinocyte cells including FEPE1L-8 cells will contribute to the development of more convenient skin models.

Conflicts of interest

There is no conflict of interest for all authors.

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