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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.

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/