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Original Article

Evaluation of the proliferative potential of skin keratinocytes and fibroblasts isolated from critical limb ischemia patients

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ABSTRACT

Impaired wound healing in critical limb ischemia (CLI) results from multiple factors that affect many cell types and their behavior. Epidermal keratinocytes and dermal fibroblasts play crucial roles in wound healing. However, it remains unclear whether these cell types irreversibly convert into a non-proliferative phenotype and are involved in impaired wound healing in CLI. Here, we demonstrate that skin keratinocytes and fibroblasts isolated from CLI patients maintain their proliferative potentials. Epidermal keratinocytes and dermal fibroblasts were isolated from the surrounding skin of foot wounds in CLI patients with diabetic nephropathy on hemodialysis, and their growth potentials were evaluated. It was found that keratinocytes from lower limbs and trunk of patients can give rise to proliferative growing colonies and can be serially passaged. Fibroblasts can also form colonies with a proliferative phenotype. These results indicate that skin keratinocytes and fibroblasts maintain their proliferative capacity even in diabetic and ischemic microenvironments and can be reactivated under appropriate conditions. This study provides strong evidence that the improvement of the cellular microenvironments is a promising therapeutic approach for CLI and these cells can also be used for potential sources of skin reconstruction.

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1. Introduction

Critical limb ischemia (CLI) is an advanced stage of lower extremity peripheral artery disease and is characterized by pain at rest and non-healing wounds. CLI patients often suffer from diabetes mellitus and end-stage renal disease, resulting in poor prognosis. Current therapies for CLI comprise amputation, bypass surgery, endovascular therapy, and pharmacological approaches. In addition to these conventional therapeutic strategies, proangiogenic gene/protein therapy and transplantation of stem cells such as mesenchymal stem cells and endothelial progenitors have been recently applied for the treatment of CLI and associated wound complications [1,2]. The impaired wound healing in CLI results from multiple factors that affect many cell types and their behavior. Normal wound healing involves many cell types, where well-coordinated cellular behavior (cell proliferation, cell migration, and extracellular matrix deposition and remodeling) is required [3]. Epidermal keratinocytes are indispensable for re-epithelialization, and dermal fibroblasts are vital for the preparation of a new substrate (granulation tissue) where keratinocytes and endothelial cells migrate to reconstruct tissues.

Keratinocyte stem cells maintain epidermal homeostasis by continuously producing functionally differentiated cells [4] and are involved in wound healing [5]. These stem cells are irreversibly converted to non-proliferative cells under some conditions [6]. Human dermal fibroblasts also have proliferative heterogeneity, and are converted to non-proliferative fibroblasts under certain conditions [7]. Although conventional and novel therapeutic approaches aim to reconstruct blood flow and improve ischemic microenvironments for reactivating skin keratinocytes and fibroblasts, it has remained unclear whether keratinocytes and

Abbreviations: CLI, critical limb ischemia; CFE, colony forming efficiency; KC, keratinocytes; FB, fibroblasts.

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fibroblasts maintain or irreversibly lose their proliferative potential under the diabetic and ischemic microenvironments. Even in these microenvironments, if these cells still possess their proliferative capacity and can be reactivated under appropriate conditions, it is convinced that the improvement of the cellular microenvironments is a promising therapeutic approach for CLI. These cells can also be applied for potential sources of skin reconstruction.

In this study, the proliferative potential of epidermal keratinocytes and dermal fibroblasts isolated from CLI patients was evaluated using the colony-formation assay. This assay is the gold standard for assessment of significant proliferative capacity of cells.

2. Materials and methods

2.1. Patients

The subjects were CLI patients with diabetic nephropathy undergoing hemodialysis. During the period from December 2014 to May 2018, their foot wounds were initially treated with percutaneous transluminal angioplasty to increase lower limb blood flow for limb salvage, and subsequently treated with minor amputation. Redundant healthy skin adjacent to foot wounds, which occurred after surgical stump closure, was examined. In the cases where autologous skin grafts after minor amputation were required, redundant donor skin taken from the trunk for skin grafting was used as the control. This study was approved by the Ethics Committees of International University of Health and Welfare (#5-16-12), Medical Research Institute, Tokyo Medical and Dental University (#02016–008 and #02018–005), and Takatsu General Hospital (#CR-1607 and #CR-18-08).

2.2. Cell culture

Epidermal keratinocytes and dermal fibroblasts were isolated from the CLI patients' skin using the explant culture method [7]. For keratinocyte isolation, the skin was cut into small pieces then plated on 60-mm cell culture dishes with keratinocyte culture medium containing a 3:1 mixture of the Dulbecco-Vogt modification of Eagle's medium (DMEM) (#11995–065; Gibco, Grand Island, NY, USA) and Ham's F12 medium (#11765–054; Gibco) supplemented with 10% fetal bovine serum (FBS) (#91760–500; Biowest, Nuaillé, France), 1.8 x 10^{-4} M adenine hemisulfate salt (#A3159; Sigma–Aldrich, St. Louis, MO, USA), 5 μg/ml insulin (#I5500; Sigma–Aldrich), 0.4 μg/ml hydrocortisone (#386698; Calbiochem, San Diego, CA, USA), 10^{-10} M cholera toxin (#190329; MP medicals, Irvine, CA, USA), and 2 x 10^{-9} M triiodothyronine (T3) (T2752; Sigma–Aldrich), as described previously [8]. For fibroblast isolation, small pieces of the skin were plated on 60-mm cell culture dishes with DMEM supplemented with 10% FBS. The skin fragments were then incubated for 2–3 weeks at 37 °C and 10% CO_2 under normoxic condition. Each cell type was selectively expanded within its specific culture medium, and migrating keratinocytes and fibroblasts from the skin fragments were collected by the treatment with 0.25% Trypsin-ethylene diamine-tetra acetic acid (EDTA) solution (#25300–054; Gibco).

Isolated keratinocytes were then cultivated on a feeder layer of mitomycin C-treated 3T3-J2 cells at 37 °C and 10% CO_2 under normoxic condition with keratinocyte culture medium as described above. Feeder cells were prepared by treating 3T3-J2 cells with mitomycin C (Kyowa Kirin co. Ltd., Tokyo, Japan) for 2 h, following by washing with phosphate buffered saline. Feeder cells were then trypsinized and seeded into cell culture dishes and plates. Keratinocytes were serially maintained by seeding 4 x 10^4 keratinocytes into 60-mm cell culture dishes with mitomycin C-treated 3T3-J2 cells at 37 °C and 10% CO_2 under normoxic condition and passaged every 7 days by the treatment with 0.05% Trypsin–EDTA. To assess the colony forming efficiency (CFE), two hundred keratinocytes a were seeded into 60-mm cell culture dishes or 6-well cell culture plates and cultured for 12 days. The cultures were fixed in 3.7% buffered formaldehyde, and stained with rhodamine B (#R6626; Sigma–Aldrich).

Isolated fibroblasts were counted and 200 cells were then seeded into 100-mm cell culture dishes at 37 °C and 10% CO_2 under hypoxic condition (2% O_2) in DMEM supplemented with 10% FBS to assess the CFE, as described previously [7]. The cultures were maintained 12 days and fixed in 3.7% buffered formaldehyde, and stained with crystal violet (#C0775; Sigma–Aldrich).

2.3. Image processing and quantification

The stained culture dishes were scanned and converted into digital images. The obtained images were binarized and the colony areas were calculated using Fiji software [9]. The number of colonies was also counted with binarized images. The CFE of keratinocytes (KC CFE) was categorized into three groups, depending on the number of colonies in an indicator dish: CFE +, ≤10 colonies; CFE ++, 10 < and ≤50 colonies; CFE +++, 50 < colonies. The CFE of fibroblasts (FB CFE) was also categorized into three groups, depending on the number of colonies in an indicator dish: CFE +, ≤50 colonies; CFE ++, 50 < and ≤100 colonies; CFE +++, 100 < colonies.

3. Results

Epidermal keratinocytes and dermal fibroblasts were isolated from the skin of CLI patients using the explant culture methods and their proliferative potentials were examined. The flow of the experimental procedures is described in Supplemental Fig. S1.

We initially examined the proliferative capacity of epidermal keratinocytes isolated from CLI patients. Keratinocytes were obtained from the foot skin of 16 CLI patients. Out of these patients, 3 CLI patients had a surgical operation for skin grafting. Keratinocytes were also obtained from the trunk skin of these 3 CLI patients. Isolated keratinocytes were then seeded into 60-mm cell culture dishes at clonal density and cultivated for 12 days to evaluate their proliferative capacity (Fig. 1a). No significant difference was found in the proliferative capacity between keratinocytes isolated from feet and trunk of all 3 patients (Figs. 1a, 2c and d). Keratinocytes isolated from the affected feet of other 13 patients were also evaluated for their proliferative capacity with the colony-formation assay. Though there were several differences among the patients, keratinocytes isolated from all 16 patients maintained proliferative capacity (Fig. 2a and c and generated colonies. Few colonies were large with smooth peripheries (Fig. 2a), a phenotype characteristic to the colony generated by a keratinocyte stem cell [10]. To confirm that isolated keratinocyte populations from the affected foot and trunk skin contain keratinocyte stem cells, we performed serial passage experiment. The proliferative capacity of keratinocytes was evaluated with the colony-formation assay after each passage. Although the colony-forming efficiency of keratinocytes showed slight decrease post several passages (Fig. 1b), the keratinocyte populations isolated from the affected foot and trunk skin maintained cells with significant proliferative capacity for at least 33 days. Collectively, these results indicate that the CLI patient's skin maintains keratinocytes with significant proliferative capacity in the affected foot and trunk.

We further examined the proliferative capacity of dermal fibroblasts isolated from CLI patients. Like keratinocytes, fibroblasts were also obtained from the foot skin of 16 CLI patients and the trunk skin of 3 CLI patients. Isolated fibroblasts were seeded into 100-mm cell culture dishes at clonal density and cultivated for 12 days under hypoxic condition to evaluate their proliferative

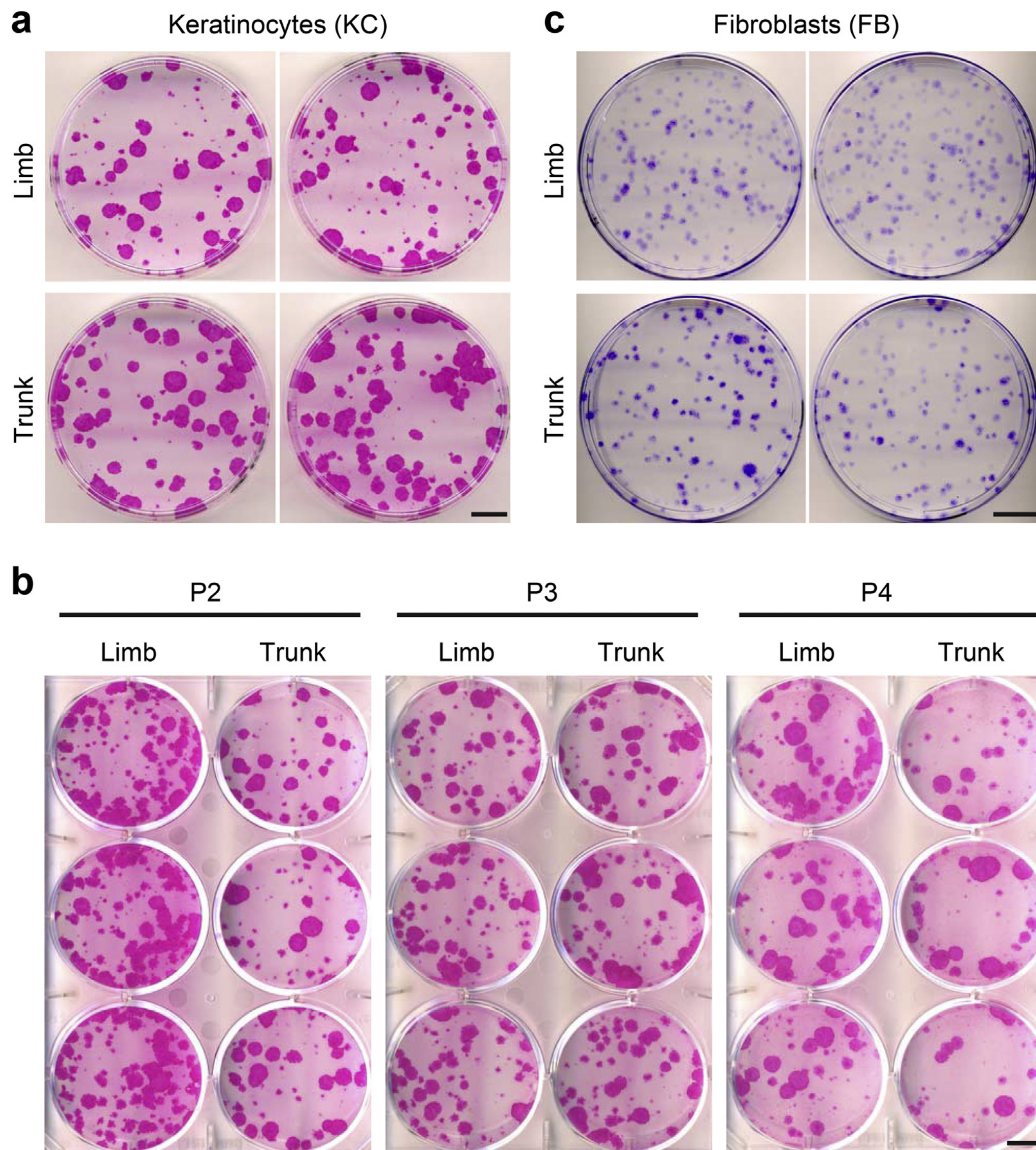


Fig. 1. Colony-formation assay of epidermal keratinocytes and dermal fibroblasts isolated from the affected limb and trunk skin of CLI patients. (a) Two hundred epidermal keratinocytes were seeded into 60-mm cell culture dishes (duplicates) and after 12 days of cultivation, the cells were stained with rhodamine B. Bar, 10 mm. (b) Colony-formation assay of keratinocytes after serial passages. Two hundred keratinocytes were seeded into each well of a 6-well plate (triplicates) and after 12 days of cultivation, the cells were stained with rhodamine B. Keratinocytes at passage 2, 3, and 4 (P2, P3, and P4) were used for the experiments. Bar, 10 mm. (c) Two hundred dermal fibroblasts were seeded into 100-mm cell culture dishes (duplicates) and after 12 days of cultivation at 2% O_2 condition, the cells were stained with crystal violet. Bar, 20 mm.

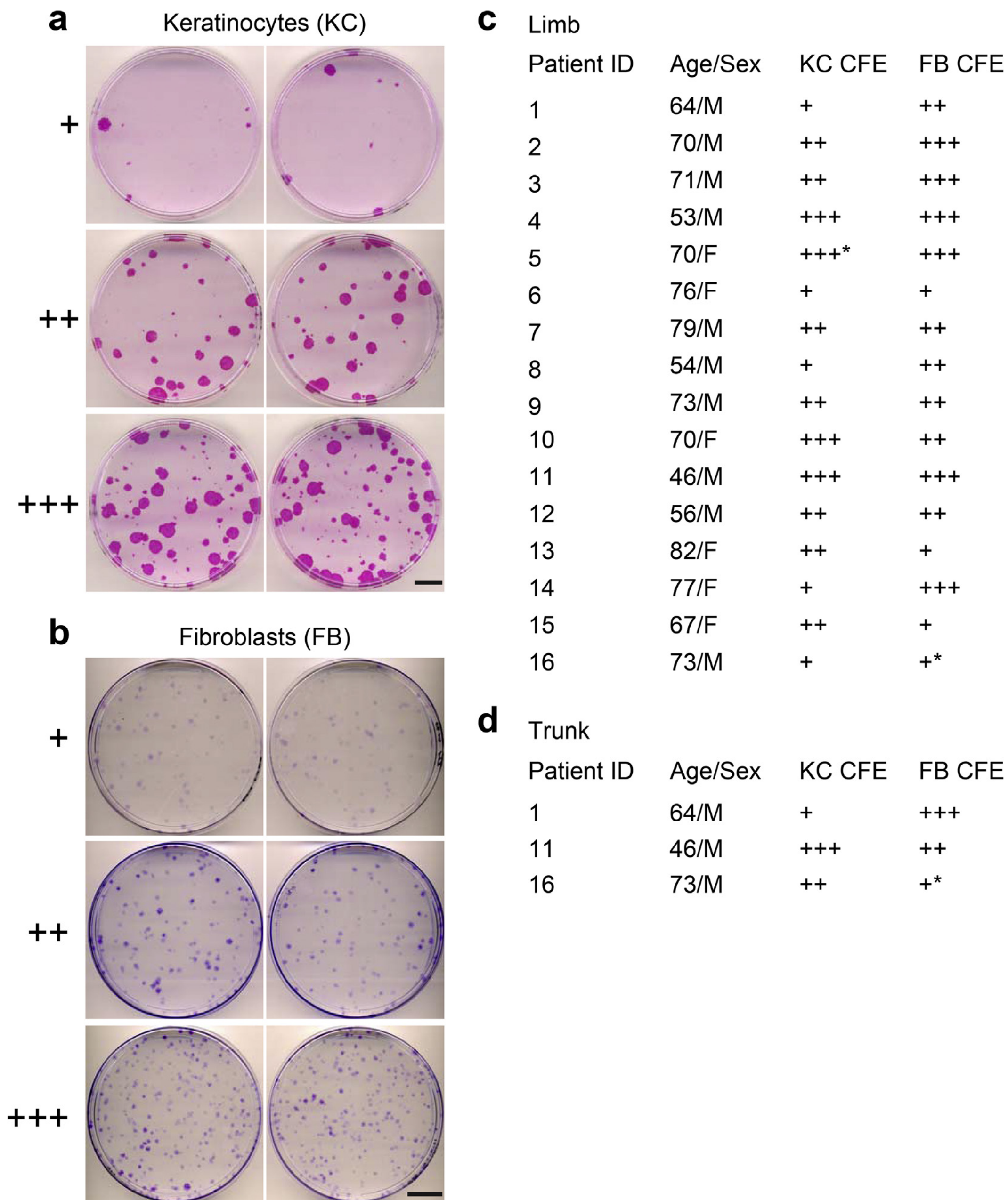
capacity [7] (Fig. 1c). Hypoxic conditions help fibroblasts to expand in low-density culture. No significant difference was found in the proliferative capacity between fibroblasts isolated from foot and trunk in all 3 patients (Figs. 1c, 2c and d). Fibroblasts isolated from the affected foot of other 13 patients were also evaluated for their proliferative capacity using the colony-formation assay. Fibroblasts isolated from all 16 patients also maintained proliferative capacity (Fig. 2b and c) and generated colonies with a proliferative phenotype [7] (Fig. 2b). Collectively, these results indicate that the CLI patient skin in the affected foot and trunk contains fibroblasts with significant proliferative capacity.

4. Case

The patient was a 73-year-old man who was undergoing treatment for type 2 diabetes since 23 years. Due to diabetic nephropathy, he was undergoing hemodialysis 4 years prior to the initial examination (Fig. S2a). He was treated at our hospital for the gangrene of his right forefoot. We attempted percutaneous transluminal angioplasty. Subsequently, the right forefoot debridement was performed. Postoperatively, negative pressure wound therapy was applied for wound bed preparation and the patient underwent skin grafting (Fig. S2b). After healing, the patient wore orthopedic shoes, and no wound recurrence was noted (Fig. S2c).

5. Discussion

This study provides strong evidence that epidermal keratinocytes and dermal fibroblasts in the affected foot of CLI patients maintain the proliferative capacity and are not irreversibly converted into a non-proliferative phenotype. These results indicate that keratinocytes and fibroblasts can be activated by improving the diabetic and ischemic microenvironments. Therefore, reconstruction of blood flow by proangiogenic gene and protein



Patient ID	Age/Sex	KC CFE	FB CFE
1	64/M	+	++
2	70/M	++	+++
3	71/M	++	+++
4	53/M	+++	+++
5	70/F	+++*	+++
6	76/F	+	+
7	79/M	++	++
8	54/M	+	++
9	73/M	++	++
10	70/F	+++	++
11	46/M	+++	+++
12	56/M	++	++
13	82/F	++	+
14	77/F	+	+++
15	67/F	++	+
16	73/M	+	+*

Patient ID	Age/Sex	KC CFE	FB CFE
1	64/M	+	+++
11	46/M	+++	++
16	73/M	++	+*

Fig. 2. Evaluation of the proliferative capacity of epidermal keratinocytes and dermal fibroblasts isolated from CLI patient skin. (a) Representative stained cell culture dishes (duplicates) for the evaluation of keratinocyte proliferative capacity. Two hundred epidermal keratinocytes were seeded into 60-mm cell culture dishes and after 12 days of cultivation, the cells were stained with rhodamine B. Bar, 10 mm. (b) Representative stained cell culture dishes (duplicates) for the evaluation of fibroblast proliferative capacity. Two hundred dermal fibroblasts were seeded into 100-mm cell culture dishes and after 12 days of cultivation at 2% O_2 condition, the cells were stained with crystal violet. Bar, 20 mm (c and d) The quantification of the colony forming efficiency (CFE) of keratinocytes and fibroblasts isolated from the limb (c) and trunk (d) skin of CLI patients. The colony number was counted and the CFE was categorized into three groups (See Materials and Methods). * indicates that the CFE was measured with the cells at passage 2 since first passage cultures were lost due to contamination.

therapies, and stem cell transplantation is a promising therapeutic approach for the treatment of CLI.

This study strongly suggests that the impaired wound healing is caused by plastic changes in behavior of keratinocytes and fibroblasts in the diabetic and ischemic microenvironments and not due to phenotypic conversion of the proliferative cells. However, proliferative capacity of keratinocytes and fibroblasts located at the leading edge of non-healing chronic wounds in the affected limbs of CLI patients was not evaluated. It is possible that the topical changes in microenvironments at the leading edge of wounds convert the proliferative keratinocytes and fibroblasts into the non-proliferative phenotype. Further investigations are needed to understand the mechanism underlying impaired wound healing in CLI patients.

Declarations Competing interests

The authors have no financial and non-financial competing interests to declare.

Ethics approval and consent to participate

This study was approved by the Ethics Committees of International University of Health and Welfare (#5-16-12), Medical Research Institute, Tokyo Medical and Dental University (#02016–008 and #02018–005), and Takatsu General Hospital (#CR-1607 and #CR-18-08).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2020.03.016.

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