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The effect of isolation and culture methods on epithelial stem cell populations and their progeny—toward an improved cell expansion protocol for clinical application

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Abstract

Background aims. The use of cultured epithelial keratinocytes in the treatment of burns and skin graft donor sites is well established in clinical practice. The most widely used culture method for clinical use was originally developed by Rheinwald and Green 40 years ago. This system uses irradiated mouse dermal fibroblasts as a feeder cell layer to promote keratinocyte growth, a process that is costly and labor-intensive for health care providers. The medium formulation contains several components of animal origin, which pose further safety risks for patients. Improvements and simplification in the culturing process would lead to clear advantages: improved safety through reduction of xenobiotic components and reduction in cost for health care providers by dispensing with feeder cells. *Methods.* We compared the Rheinwald and Green method to culture in three commercially available, feeder-free media systems with defined/absent components of animal origin. *Results.* During the isolation process, short incubation times in high-strength trypsin resulted in increased numbers of liberated keratinocyte stem cells compared with longer incubation times. All three commercially available media tested in this study could support the expansion of keratinocytes, with phenotypes comparable to cells expanded using the established Rheinwald and Green method. Growth rates varied, with two of the media displaying comparable growth rates, whereas the third was significantly slower. *Discussion.*. Our study demonstrates the suitability of such feeder-free media systems in clinical use. It further outlines a range of techniques to evaluate keratinocyte phenotype when assessing the suitability of cells for clinical application.

Key Words: *burns, cell therapy, keratinocyte, skin, sprayed autologous keratinocytes, stem cells, wound healing*

Introduction

Severe burns continue to present a significant clinical challenge. At present, consensus guidelines for treatment regimes for such injuries are lacking, resulting in great disparity in treatment modalities between countries [1]. The application of cultured epithelial keratinocytes is well established as a means of augmenting wound healing [2–11], although the exact mechanism through which the cells contribute to healing remains uncertain. The use of confluent cell sheets was first developed in the early 1980s, when several articles described the treatment of patients with severe, full-thickness burns with confluent sheets of cultured autologous epithelial keratinocytes [12,13]. The long-term benefit of using such confluent sheets has remained controversial because they have shown in clinical practice to be fragile and prone to infections, resulting in poor engraftment [9]. More commonly, autologous epithelial keratinocytes are now transplanted in single-cell suspension or as subconfluent cultures on a carrier matrix [9,14,15]. Which treatment method is used depends on the surgical team's choice and the availability of specialist units that prepare cells for transplantation. Whether transplanted in suspension or on a carrier, keratinocytes used clinically must maintain good re-implantation potential—that is, engraft permanently, produce progeny and construct an effective epidermis [16]. It is thought that keratinocyte stem cells (SCs)

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and their actively cycling transiently amplifying (TA) progeny harbor significant long-term tissue regenerative capacities, whereas differentiating cells do not contribute to the regeneration of a functional epidermis [16,17]. Thus, retaining the SC and TA phenotypes during keratinocyte culture is most likely to result in the successful contribution of implanted cells to the development of a robust epidermis.

Methods used to support the proliferation of keratinocytes in culture for clinical application have relied predominantly on xenogeneic products. Irradiated 3T3 mouse fibroblasts were first used by Rheinwald and Green 40 years ago as a feeder layer for the culture of keratinocytes [18]. Application of the Rheinwald and Green (R&G) culturing process consistently generates rapidly expanding keratinocyte populations that maintain good re-implantation potential to meet clinical demand [16]. Although there are clear advantages to the culture of keratinocytes using irradiated murine fibroblasts, there are a number of limitations. Concerns have been raised regarding the uptake of irradiated DNA or xenobiotic antigens into keratinocytes and an associated risk of immunologic activation and cell destruction upon implantation [19,20]. In addition, the requirement for a radioactive source for lethal irradiation of murine fibroblasts poses a substantial logistical and financial burden on health care providers. An improved culturing protocol, without feeder cells and xenobiotics in the media, suitable for clinical application of cells, would improve safety for patients and reduce health care costs.

Significant progress has been made in developing media systems that reduce the need for animal-derived products [21–25]. We previously demonstrated the use of non-irradiated autologous human fibroblasts to replace murine fibroblasts [26]. However, there is only one report to date that describes the clinical use of keratinocytes expanded using a xenobiotic-free medium system for burn injuries [25]. The cells used in this unit are allogeneic keratinocytes isolated from neonatal foreskin. In our own experience, using a large porcine model of wound repair, allogeneic keratinocytes do contribute to wound healing, but significantly less than do autologous cells [27]. The requirement for improved culturing methods for clinical application is therefore clear.

Typically, the application of cultured keratinocytes to burns patients with full-thickness wounds takes place between 4 and 20 days after admission [10,15,28]. Ideally, any culturing method that is to be used clinically will yield significant numbers of cells in a comparable time period.

Here we report the effect of isolation and culture methods on the successful expansion of epithelial SCs and their progeny, comparing three commercially available feeder-free media systems with culture in R&G medium with the specific aim of producing cells suitable for clinical treatment of extensive cutaneous defects.

Materials and Methods

Cell isolation and culture

Primary human keratinocytes were isolated and cultured as previously described [29]. Briefly, skin was obtained with patient consent and full approval from the National Regional Ethics Service (REC: 06/Q1907/81) from discarded tissue during routine surgical procedures. The dermis and epidermis were separated using dispase (4 mg/mL, Invitrogen, Paisley United Kingdom) by incubation for 30 min at 37°C. Keratinocytes were released from the epidermis by incubating in 0.5% trypsin 1:250 (Gibco Paisley, United Kingdom) at the following time intervals for initial testing: 5, 10, 20 and 30 min at 37°C. Subsequently, keratinocytes were released by 10-min incubation in trypsin. Cells were seeded at a density of 2×10^4 cells/cm^2 [30] in the testing medium formulations (Table I), which were as follows: (R&G)—R&G medium consisting of Dulbecco's modified Eagle's medium and Ham's F12 medium (Invitrogen) at a 3:1 ratio, supplemented with 10% Fetal calf serum (FCS) FCS (Gibco), 10 ng/mL human recombinant EGF Epithelial growth factor (EGF) (Invitrogen), 10 nmol/L cholera toxin (Sigma Sigma-Aldrich, Gillingham, United Kingdom) and 0.4 mg/mL hydrocortisone (Sigma-Aldrich); Celloneer KC Defined Medium (CKCD)—CKCD with supplements (PAA Yeovil, United Kingdom), otherwise known as CnT-07 medium (CellnTech, Bern, Switzerland); EpiLife Defined Growth Supplement (EDGS)—defined medium EpiLife with EDGS supplement (Gibco); S7—defined medium EpiLife with S7 supplement (Gibco). Preliminary experiments were also performed using Keratinocyte Serum-Free Medium (Gibco) and Keratinocyte Growth Medium 2 (Promocell, Heidelberg, Germany) growth media. However, growth in these media was inadequate to be included in this study because cell expansion after primary isolation to the first passage required more than 3 weeks. This is not suitable for clinical use of cells, and thus these media were excluded from subsequent experiments. For growth in EpiLife

Table I. Summary of absence/presence of feeder cells and xenobiotics in the four media formulations.

	R&G	CKCD	EDGS	S7
Feeder cells	Yes	No	No	No
Xenobiotics	Yes	Defined	Defined	No