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Quantitative Assay for Quality Assurance of Human Cells for Clinical Transplantation

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Transplantation of human cells after isolation and culture has become an important alternative for treatment of acute or chronic skin wounds. To increase the efficacy and reduce cost for transplantation of skin cells, more efficient and accurate techniques for evaluation of cell proliferation are needed. Hemocytometer counts provide a valid assessment of cell proliferation and viability, but they are very labor intensive and require removal of the cells from their substrate. In this study, hemocytometer counts were compared with a fluorometric assay ($n = 21$ per condition) that uses the commercially available reagent alamarBlue™, which is reduced to a fluorescent substrate by cellular dehydrogenases. Human epidermal keratinocytes were inoculated at 200, 600, 2000, and 6000 cells/cm^2 incubated for 6 days in modified MCDB 153 medium. Alamar Blue™ was incubated with cells for 2 h at 37°C, and fluorescence was measured with a microplate reader at 590 nm. Hemocytometer counts ($\times 10^{-4}$) from the respective cell inoculation densities were 0.30 ± 0.04, 1.07 ± 0.10, 6.37 ± 0.62, and 16.99 ± 0.96. Fluorescence values ($\times 10^{-3}$) for the respective inoculation densities were 0.14 ± 0.01, 0.34 ± 0.02, 1.20 ± 0.09, and 1.79 ± 0.12. Regression analysis showed a statistical significant ($p < 0.0001$) correlation ($r^2 = 0.87$) between cell counts and optical density from the alamarBlue™ assay. These data demonstrate that alamarBlue™ provides a valid substitute for cell counts to assess cell proliferation before clinical transplantation of engineered skin. AlamarBlue™ also allows repeated, nondamaging assessment of living cells over time. These advantages are expected to increase the validity and reliability of quality assurance standards for transplanted skin cells, and to increase the efficacy of healing of cutaneous wounds.

Key words: Cultured keratinocytes; Skin substitutes; Wound healing; Burns; Chronic wounds

INTRODUCTION

Assurance of cellular viability in transplantation of engineered tissues is definitive to their survival, engraftment, and function. For tissue engineering applications, assessment of viability must be rapid, accurate, and simple to perform. Viability may be measured as general or specific metabolic activities in growth-arrested cells, or as DNA synthesis or mitosis in proliferating cells. Several measures of metabolic viability have been reported, including oxygen consumption (18,37), MTT conversion (22,31), lactate dehydrogenase (19,23), or Alamar Blue conversion (21,38,39). Cellular proliferation may be measured directly either by assessment of DNA synthesis by incorporation of tritiated-thymidine or bromodeoxyuridine, or by hemocytometer counts of cell numbers. Automated cell counting may be performed with machines that count particles (Coulter counters), or that record events by scattered or emitted fluorescent light from a laser light source in a laminar-flow stream of fluid (i.e., flow cytometers). Indirect measures of cellular proliferation include proliferating cell nuclear antigen (PCNA) (28,43), p63 (20,26,30), Ki67 (1,36), or ratios of acridine orange to propidium iodide (3,29,45). Uptake of dyes, such as neutral red or crystal violet, have been reported to correlate with cell numbers (5), but the chemical stability of these dyes can introduce variability in the accuracy of data. Exclusion of the dye, trypan blue, has been reported historically as an index of viability (25,32,33), but it determines only whether the plasma membranes of cells are intact, and does not measure either intermediary metabolism or DNA synthesis. Selection of an assay that is accurate, sensitive, reliable, and simple requires multiple considerations of both the cells to be evaluated and the assay parameters. Proliferating cells, such as dermal or epidermal skin

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cells, must retain some mitotic potential to form stable and functional tissue after transplantation (6–8). In these examples, the ultimate objective is to regulate cellular physiology as closely as possible to the natural tissue in the uninjured condition.

Previous studies from the authors' laboratories have described determinations of cellular viability by hemocytometer counts, MTT conversion, and incorporation of bromo-deoxyuridine (9–11,41). Cultured human epidermal keratinocytes and fibroblasts have been transplanted successfully to patients with burns (12–14), chronic wounds (15), and congenital giant melanocytic nevi (35). The purpose of the current experiments was to adapt available techniques for assay of cellular viability by alamar Blue™ conversion, and to correlate those measurements with cell numbers as determined by hemocytometer counts. Hypothetically, the availability of a simple and reproducible assay with the accuracy and reliability of hemocytometer counts may reduce labor intensity, conserve resources, and improve the predictability of performance of human cells for clinical transplantation.

MATERIALS AND METHODS

Cell Culture

Human epidermal keratinocytes were established from primary cultures of surgical discard skin obtained with approval of the University of Cincinnati Institutional Review Board. The cells were inoculated into 75-cm^2 flasks (Corning) at 2000 cells/cm^2, containing 15 ml modified MCDB 153 (5,16), and incubated at 37°C, 5% CO_2 (17). After 6 days they were harvested and the cells were inoculated into six-well dishes (Corning) containing 3 ml/well at the following densities: 200, 600, 2000, or 6000 cells/cm^2. Each condition was repeated 21 times ($n = 21$). The cells were inoculated in, and changed with, modified MCDB 153.

Alamar Blue Assay

On incubation day 6, the medium was aspirated and replaced with 3 ml per well of 10% solution of alamar Blue™ (Biosource International, Inc., Camarillo, CA) in unsupplemented serum-free MCDB 153. Cells were incubated for 2 h at 37°C, 5% CO_2. The plates were then read with the fluorescence Multi-Well Plate Reader SpectraMax Genesis (Molecular Devices, Inc., Sunnyvale, CA) at 530 nm excitation, 590 nm emission. The emission spectra of the oxidized and reduced forms of alamar Blue™ are both at 590 nm, as shown in Table 1 (2,34).

Cell Counts

To determine cell numbers, cells were trypsinized from individual wells, transferred to medium containing 10% fetal bovine serum to neutralize the trypsin, and counted with a standard hemocytometer.

Table 1. Wavelength (nm) Maxima for Oxidized and Reduced alamarBlue™ in Fluorescence and Transmission Spectrometry

alamarBlue™ Oxidation State	Fluorescence		Absorbance (Optical Density)
	Excitation	Emission	
Oxidized	530	590	600
Reduced	530	590	570

Data and Statistical Analyses

Experimental conditions were performed in replicates of 3–6 per experiment, and repeated to generate $n = 21$ per condition. Cell numbers were expressed as cellular inoculation and harvest densities (cells/cm^2). Alamar Blue conversion was expressed as relative fluorescence units at 590 nm. Regression analysis was performed for correlation of cellular inoculation densities to cellular harvest densities, cellular inoculation densities to relative fluorescence units at 590 nm, and of cellular harvest densities to relative fluorescence units at 590 nm.

RESULTS

Phase contrast photomicrographs of normal human epidermal keratinocytes before and after exposure to alamar Blue™ are shown in Figure 1. No morphologic changes occurred after 2-h exposure to the alamarBlue™ dye.

Quantification of growth of normal human epidermal keratinocytes by standard hemocytometer counts is shown in Figure 2A. Cellular harvest density increased as a function of increasing cellular inoculation density. Correlation of inoculation and harvest densities was statistically significant ($p < 0.0001$) for $r^2 = 0.93$.

Quantification of growth of normal human epidermal keratinocytes by measurement of relative fluorescence units in the alamarBlue™ assay is shown in Figure 2B. Fluorescence increased as a function of increasing cellular inoculation density. Correlation of inoculation and harvest densities was statistically significant ($p < 0.0001$) for $r^2 = 0.88$.

Correlation of cellular harvest density to relative fluorescence units is shown in Figure 3. Correlation of harvest density to fluorescence was statistically significant ($p < 0.0001$) for $r^2 = 0.87$. The regression line from the recorded data generated the following formula for prediction of cellular harvest density from fluorescence values:

$$\text{predicted } \log_{10} (\text{cellular harvest density}) = 0.15949 + 0.59201\ (\log_{10} (\text{fluorescence units}) \quad (1)$$

DISCUSSION

Quantification of cell division is a fundamental measure of the viability of cellular populations for perfor-

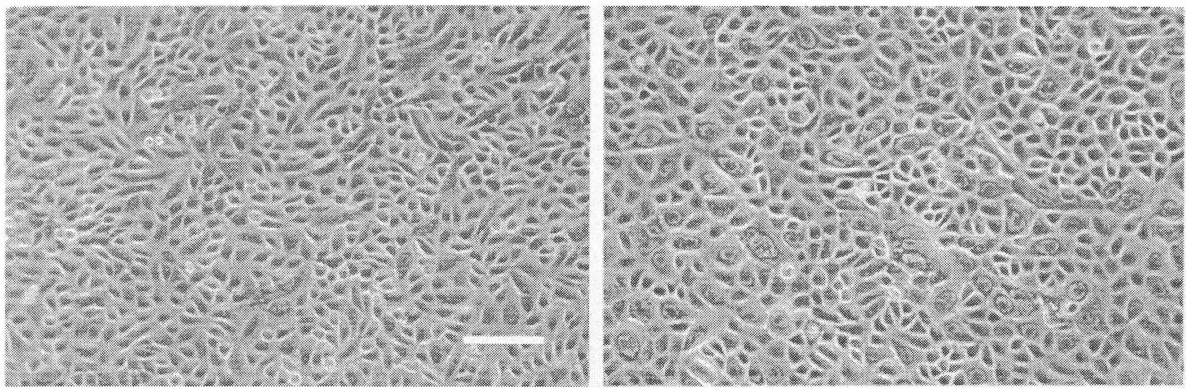

Figure 1. Photomicrographs of normal human epidermal keratinocytes before (left) and after (right) exposure for 2 h to alamar Blue™ dye. No morphologic changes were observed. Scale bar: 0.1 mm.

mance of preclinical experiments, or for clinical transplantation. Accuracy of quantification is perhaps the most important factor among several considerations, including simplicity, speed, cost-effectiveness, continuous availability, and reproducibility. The data presented here demonstrate that measurement of fluorescence units after biochemical reduction of the alamarBlue™ dye to a red fluor makes a valid prediction of cellular harvest density without trypsinization or cellular counting.

The alamarBlue™ assay may be performed in either fluorescence or absorbance spectrophotometric mode. However, in absorbance mode the maximum absorbance wavelength of the oxidized dye (OD_{600}) generates a very high background value (>60%) at the maximum absorbance wavelength of the reduced dye (OD_{570}). Conversely, the maximum fluorescence wavelength of the oxidized and reduced dyes are both at 590 nm, at which wavelength the oxidized dye generates less than 10% of the signal of the reduced dye. Therefore, fluorescence mode for the alamarBlue™ assay has much greater sensitivity due to lower background emission of the oxidized dye.

Most of the biochemical reduction of the alamar Blue™ is believed to occur in the electron transport system of cellular respiration. Biochemical reduction of both alamarBlue™ and MTT occurs by reaction of the

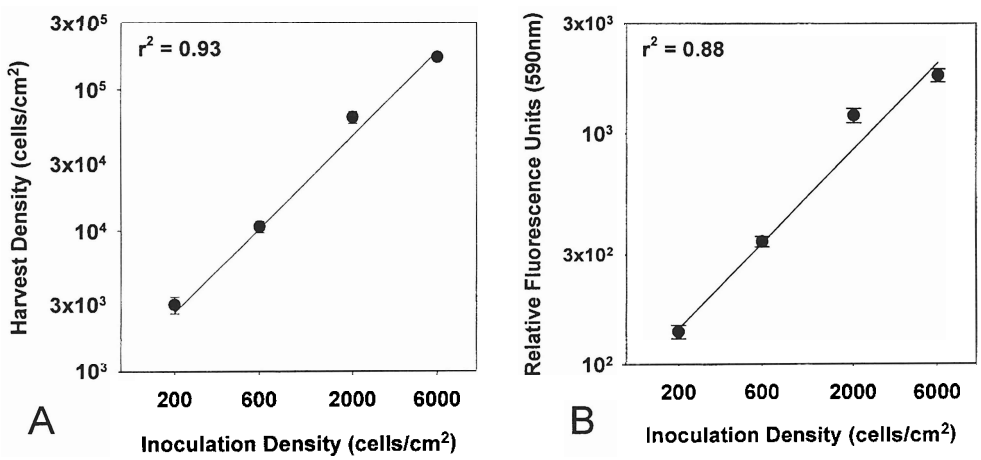

Figure 2. Correlation of cellular inoculation density with cellular harvest density, and with relative fluorescence units in the alamarBlue™ assay. (A) Numbers of normal human keratinocytes were counted on a standard hemocytometer at inoculation and at harvest by trypsinization after 6 days of incubation. Correlation ($r^2 = 0.93$) was statistically significant ($p < 0.0001$). (B) Correlation of relative fluorescence units to cellular inoculation density. Fluorescence values were determined at 590 nm in a spectrofluormetric plate reader after 6 days of incubation, and cellular inoculation densities were by hemocytometer counts. Correlation ($r^2 = 0.88$) was statistically significant ($p < 0.0001$).

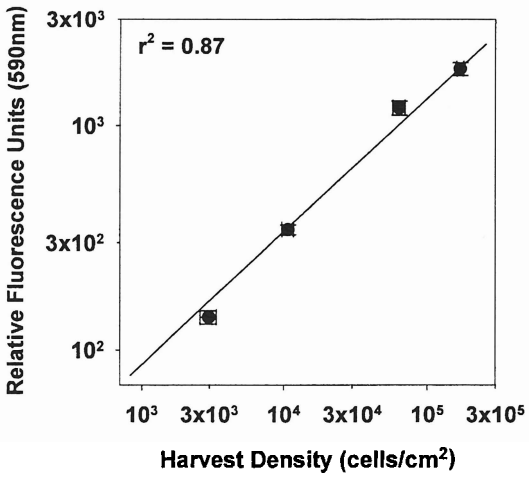

Figure 3. Correlation of cellular harvest density with relative fluorescence units in the alamarBlue™ assay. Cellular harvest densities were determined by hemocytometer counts, and fluorescence values were determined at 590 nm in a spectrofluormetric plate reader after 6 days of incubation. Correlation ($r^2 = 0.87$) was statistically significant ($p < 0.0001$).

oxidized dyes with $2H^+ + 2e^-$ to generate the reduced dye. However, the potential for the reaction depends on the electronegativity of the substrate. Because alamar Blue™ is more electronegative than MTT, it can be reduced by cytochromes as well as by FMN, FAD, NAD, and NADPH. Therefore, the biochemical sensitivity of the alamarBlue™ assay is greater than the MTT assay.

Dye uptake by cells in monolayer has also been reported as an index of cell numbers or cellular viability. Chromatic staining of cells with crystal violet after chemical fixation, followed by release of the stain and spectophotometry has been reported (27,42) as an index of cell numbers, but is subject to errors resulting from the spontaneous precipitation of the stain from solution, and the photodegradation of the stain. Multiple studies in this laboratory (not shown) failed to demonstrate reproducibility on an absolute, quantitative scale, which compromises comparisons among experiments. Uptake of neutral red by living cells (4,40,44) is more reliable and reproducible than crystal violet, but is a terminal assay from which cells cannot recover. By comparison, the alamarBlue™ is nondestructive, and allows recovery of living cells for subsequent observation.

Estimation of cellular population doublings (PDs) is a valuable index that may be calculated from direct determination of cell numbers at inoculation and harvest by the formula:

$$PDs = [\log_2(\text{cellular harvest density}/\text{cellular inoculation density})] \quad (2)$$

Because of the validity of the correlation between cellular harvest density and fluorescence in the alamar Blue™ assay, fluorescence values may be converted to PDs by substitution of the predicted value for cellular harvest density from the alamarBlue™ assay (formula 1 above). These calculations assume a constant colony-forming efficiency for multiple inoculation densities performed simultaneously. Also, as shown in Figure 2A, there may be a slightly disproportionate increase in cell numbers harvested as a function of an increase of inoculation density. For example, an approximately 30-fold increase of cells harvested results from an inoculum of 200 cells/cm^2, but a somewhat greater than 30-fold increase of cells harvested results from an inoculum of 6000 cells/cm^2. This may represent higher colony-forming efficiency or higher growth rate at the higher inoculation density. In either case, the greater cell harvest density may be attributed to conditioning of the medium with autocrine growth factors (24) as a function of cell density. This discrepancy in harvest density versus inoculation density is independent of the assay format, and therefore does not affect the correlation between the measurement end points.

These several advantages of the alamarBlue™ assay for cellular viability offer savings of resources, and high validity for preclinical and clinical studies of normal human cells for transplantation or other diagnostic or therapeutic purposes. These advantages are expected to contribute to more rapid advancement of studies for tissue engineering of human skin for cutaneous wounds, and of other tissues to reduce morbidity for qualified populations of patients.

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