A New, Simple, Nonradioactive, Nontoxic In Vitro Assay to Monitor Corneal Endothelial Cell Viability

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Purpose. This study was designed to determine whether Alamar blue could be used to evaluate corneal endothelial cell viability in vitro.

Methods. Alamar blue incorporates a proprietary redox indicator that changes color in response to metabolic activity. Primary rabbit endothelial cells were subcultured on 96-well plates at densities ranging from 1,250 to 40,000 cells per well. After 12 hours' incubation, Alamar blue was added to each well and absorbance measured hourly from 1 to 9 hours. Sodium azide-killed cells were used as a control. Alamar blue conversion was also compared with [3H]-thymidine incorporation in the presence or the absence of mitomycin C.

Results. Alamar blue reduction demonstrated endothelial cell viability at all cell concentrations compared with that in killed-cell controls. The reduction varied proportionately with cell number and time, showing clearly significant differences. Conversely, [3H]-thymidine uptake demonstrated minimal DNA synthesis and little or no ability to distinguish cell number or viability.

Conclusions. Alamar blue reduction measures endothelial cell viability and can readily differentiate cell concentrations. It demonstrates several advantages over [3H]-thymidine: It can assay nonproliferating endothelial cell metabolism, it allows rapid assessment of large numbers of samples, it can differentiate endothelial cell concentrations, it is nontoxic, it is nonradioactive and allows for simple disposal, it is less costly, and it allows for continuous monitoring of endothelial cell metabolism and viability.

The ability to measure corneal endothelial cell viability in vitro accurately is important for applications that include measuring the toxicologic effects of intraocular drugs and irrigating solutions and basic physiologic studies of endothelial cell metabolism. Corneal endothelial cell viability has special importance in eye banks, for which assessment of cell viability is crucial to the development of storage media for donor corneas. The ideal method of measuring corneal endothelial cell viability would not be toxic to the endothelial cell, allowing continuous or repeated measurements; would have a range of sensitivity that allows measurement of degrees of viability; would not be dependent on cell division; and would be convenient and safe.

Many methods, all of which have significant limitations, have been used to measure the viability of endothelial cells. The Alamar blue assay (Accumed International Inc., Westlake, OH) is a proprietary assay designed to measure quantitatively cell proliferation, cytotoxicity, and viability by incorporating resazurin and resorufin as fluorometric-colorimetric oxidation-reduction indicators that fluoresce and change color in response to chemical reduction resulting from cell metabolism. Alamar blue has certain properties that make this assay attractive. It is soluble in media, stable in solution, minimally toxic to cells, and produces changes that are easy to measure. Alamar blue has been used as a measure of cell viability in tumor necrosis factor (TNF)-hypo-sensitive cell lines, in studies of apoptotic neuronal death, and in studies of lym-
phocyte proliferation. In this report, we describe the use of the Alamar blue assay to measure rabbit corneal endothelial cell metabolism and compare the results with those of $[^{3}H]$-thymidine incorporation. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

MATERIALS AND METHODS

Rabbit Corneal Endothelial Cell Culture

Corneal endothelial (CE) cells were obtained from euthanized weanling rabbits by exposing the excised corneas to 0.83% trypsin with 0.016 M EDTA for 30 minutes. The endothelium was flushed with phosphate-buffered saline (PBS) to remove loosened cells. The CE cells were washed twice in PBS and resuspended in Gibco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum. The cells were placed into Corning T-75 flasks (Corning Glass Works, Corning, NY) in 100-μl aliquots. The plate was then incubated at 37°C in 5% CO$_2$ for 10 days, with media changes at days 1 and 7. At day 10, the cells were confluent, yielding 450,000 CE cells per flask.

Alamar Blue Assay

Comparison of Cell Number and Incubation Time. The CE cells were removed from the flasks with 0.83% trypsin, centrifuged, washed twice in PBS, and adjusted to 40,000 cells per 100 μl. Serial dilutions of cells ranging from 40,000 to 1,250 cells per well were placed in 96-well microplasty reader plates (Corning Glass Works, Corning, NY) in 100-μl aliquots. The plates were incubated for 12 hours to allow the cells to adhere. The media were then removed and 100 μl fresh medium containing 9% Alamar blue reagent was added to each well, according to the manufacturer's recommendations. Alamar blue is provided as a sterile, ready-to-use, multicomponent solution that is diluted directly in culture medium. The plate was then incubated at 37°C. The absorbance was read on a Titertek spectrophotometer at 570 nm (reduced) and 595 nm (oxidized) every hour for 9 hours and again at 24 hours.

Because of overlap in the absorption spectra of the oxidized and reduced form of Alamar blue, an equation derived by Willard et al., and provided by the manufacturer, allows calculation of the percentage of reduction:

\[
\% \text{ Reduced} = \frac{(117,216)A_{570} - (80,586)A_{600}}{(155,677)A'_{600} - (14,652)A'_{570}} \times 100
\]

wherein 117,216 = molar extinction coefficient of Alamar blue in the oxidized form at 600 nm; 80,586 = molar extinction coefficient of Alamar blue in the oxidized form at 570 nm; 14,652 = molar extinction coefficient of Alamar blue in the reduced form at 570 nm; $A_{600}$ = absorbance of test wells at 600 nm; $A_{570}$ = absorbance of test wells at 570 nm; $A'_{600}$ = absorbance of negative control wells at 600 nm; $A'_{570}$ = absorbance of negative control wells at 570 nm.

Statistical analysis of individual assays was performed using analysis of variance with a multiple comparison test using Tukey's Studentized range and Student's t-test. P values were calculated using the t-test with a two-tailed distribution and unequal variance.

Effect of Sodium Azide. To test the effect of the metabolic poison sodium azide, CE cells were suspended in medium containing 1.15% sodium azide and dispensed to microculture plates. After 11 hours, the medium was replaced with 5% sodium azide for 1 hour. The azide was then removed and replaced with 100 μl of fresh medium containing 9% Alamar blue reagent. The plate was incubated and the absorbance read to determine Alamar blue reduction.

Comparison With $[^{3}H]$-Thymidine Incorporation. In three experiments, after adding Alamar blue to the 96-well plate, 1 μCi of $[^{3}H]$-thymidine was added to each well and incubated as before. After colorimetric assay for Alamar blue reduction at 6 hours and at 24 hours, the plates were frozen at −80°C and thawed. The lysates were harvested on glass fiber filters, and $[^{3}H]$-thymidine incorporation was determined by scintillation counting. To block DNA transcription in control wells, 33 μg/ml mitomycin C (Sigma Chemical) was added at the time of seeding to 35 of the 70 wells used. It was left in the wells for 20 minutes at 37°C. $[^{3}H]$-thymidine and Alamar blue were added after the 12-hour attachment incubation. Thus, one half of the plate was exposed to mitomycin C, and the entire plate had $[^{3}H]$-thymidine and Alamar blue in each well. Each half of the plate contained 5 wells of every cell concentration.

RESULTS

Because metabolic activity in the Alamar blue assay can be detected by either the absorbance at 570 nm or by calculating the percentage of reduction, we compared the two methods on the same cultures (Fig. 1). Measuring Alamar blue absorbance at 570 nm shows statistically significant differences between every cell concentration at 4 hours ($P < 0.05$; Fig. 1A). To reduce the effect of overlap of the oxidized spectra, the percentage of reduction was calculated (Fig. 1B), resulting in significant differences ($P < 0.001$) at 2, 3, 4, and 5 hours at every cell concentration. Both procedures reveal that a plateau is reached at higher...
In Vitro Assay of Corneal Endothelial Cell Viability

0.6-
0.5-
0.4-
0.3-
0.2-
0.1-
0.0-

FIGURE 1. The metabolic conversion of Alamar blue correlates with cell number and incubation time. (□), 40,000 cells per well; (○), 20,000 cells per well; (○), 10,000 cells per well; (△), 5,000 cells per well; (▽), 2,500 cells per well; (▷), 1,250 cells per well; (<), no cells per well. (A) Absorption of Alamar blue at 570 nm measured from 2 to 9 hours. (B) Percentage of reduction of Alamar blue after correcting for the effect of overlap of the oxidized spectra. The absence of error bars indicates that the error was smaller than the symbol used.

cell numbers and longer culture times. Comparable results were obtained in numerous assays; therefore, only a representative experiment is shown.

In vivo, only rare mitotic CE cells are observed rarely, so that measurements of CE cell activity based on macromolecular synthesis examine the activity of cells not representative of the population. However, these assays are convenient, and their use has been reported. For purposes of comparison, we have examined Alamar blue and [3H]-thymidine, and added mitomycin C to some cultures to block DNA transcription and mitosis to mimic the nonmitotic state observed in vivo. [3H]-thymidine incorporation was measured at 6 and 24 hours, with mitomycin C as a control (Fig. 2). The [3H]-thymidine counts were very low, with broad scatter, and exhibited poor correlation with cell number at 6 and 24 hours (Fig. 2A). A small difference was observed between wells, with and without mitomycin C, indicating that some DNA synthesis was occurring (Fig. 2B). Even wells seeded with subconfluent numbers of CE cells showed minimal [3H]-thymidine incorporation. In contrast, concurrent Alamar blue reduction at 6 hours demonstrated that a substantial level of active metabolism was occurring proportionate to cell number, despite inhibition of DNA synthesis with mitomycin C (Fig. 2C).

The effect of mitomycin C on Alamar blue reduction was examined more closely in Figure 3. After treatment with mitomycin C (Fig. 3B), the CE cells continued to reduce Alamar blue, demonstrating a strong correlation between reduction and either cell number or culture time. Less conversion of Alamar blue was found than in parallel cultures not treated with mitomycin C (Fig. 3A). This difference is caused in part by the lack of increase in cell number following the mitomycin C treatment.

FIGURE 2. [3H]-Thymidine incorporation, with and without mitomycin C, at 6 and 24 hours. (A) Cells without mitomycin C counted at 6 (○) and 24 (□) hours. (B) Cells with (○) and without (□) mitomycin C counted at 24 hours. (C) Alamar blue reduction at 6 hours in the presence of mitomycin C.
FIGURE 3. Percentage of reduction of Alamar blue, with and without mitomycin C. (•), 40,000 cells per well; (◇), 20,000 cells per well; (○), 10,000 cells per well; (△), 5,000 cells per well; (▽), 2,500 cells per well; (◇), 1,250 cells per well. (A) Percentage of reduction of Alamar blue without mitomycin C. (B) Percentage of reduction of Alamar blue with mitomycin C. The absence of error bars indicates that the error was smaller than the symbol used.

Because Alamar blue is used to measure metabolic activity, the effects of sodium azide were examined. Sodium azide, a heme poison, inhibits cytochrome-c oxidase, which catalyzes the final reduction of O₂, and also reduces Alamar blue. Figure 4 shows complete inhibition of Alamar blue reduction by sodium azide at all cell concentrations and demonstrates that Alamar blue reduction is dependent on an active electron transport chain.

FIGURE 4. Inhibition of reduction of Alamar blue with sodium azide as killed-cell control. (○), 40,000 cells per well; (◇), 20,000 cells per well; (○), 10,000 cells per well; (△), 5,000 cells per well; (▽), 2,500 cells per well; (◇), 1,250 cells per well. Solid lines represent culture without sodium azide, dashed lines those with sodium azide. The absence of error bars indicates that the error was smaller than the symbol used.

DISCUSSION

The results of this study demonstrate distinct advantages of Alamar blue as an agent in the assay of corneal endothelial cell metabolism and viability. Together, the mitomycin C, sodium azide, and [³H]-thymidine results show that Alamar blue is a superior measure of the energy-producing metabolism of mitotically quiet and dividing CE cells. Monitoring of endothelial cell culture metabolism can be maintained for at least 9 hours. Quantitative differentiation of cell densities from 1,250 cells/well to 40,000 cells/well at 2 to 5 hours' incubation was possible, showing significant differences. This time frame is convenient for laboratory experiments.

Alamar blue reduction reached a plateau with high cell concentrations or prolonged incubation times, caused in part by a second redox step that has been reported in other systems to result in a colorless compound. It is also consistent with our observation that seeding 20,000 CE cells per well is sufficient to yield confluent cultures quickly; consequently, addition of 40,000 cells per well would not be expected to yield significantly higher results. To adjust the incubation period within the linear range of Alamar blue reduction, fewer cells would be used. The incubation conditions used in this study were ideal to differentiate up to 20,000 CE cells per well for up to 9 hours, with the ability to differentiate all cell concentrations in all assays at the 4-hour incubation time.

Various approaches to assessment of corneal endothelial cell viability have been taken. Morphologic methods, including ultrastructural studies, are used but require substantial effort, highly skilled personnel, and expensive equipment; do not yield easily quantifiable results; and destroy the sample. Cell membrane integrity can be assessed by dye uptake and vital stains. However, they are not as direct a measure of viability and are terminal assays that destroy the cell or interfere with its function. Measurements of glucose uptake and lactic acid release into incubation media allow continuous monitoring of endothelial cell viability, but such methods are labor intensive and relatively insensitive. The reduction of tetrazolium salts by mitochondrial succinate dehydrogenase activity uses hazardous reagents and requires washing, fixing, and extraction steps that destroy the cell. Corneal deturgescence has been used to study endothelial viability in vitro. Its usefulness is limited because it is an indirect test lacking sensitivity, requires whole cornea preparations, and is subject to many methodologic errors. Transendothelial cell potential using a whole cornea preparation is used to measure endothelial cell pump function. However, this is primarily a physiologic laboratory test unsuitable for routine use, and the cornea cannot be used after measurement.
Transplanting the endothelium is the ultimate test of its viability, but it is an impractical test for obvious reasons, and it uses the cornea.

Human endothelial cell proliferation measured by [3H]-thymidine incorporation has been studied as a test of viability in corneal endothelial cell cultures. However, cell division is not a normal physiologic response of the human corneal endothelial cell, necessitating use of growth factors, mitogens, or mechanical wounding to observe significant incorporation. Assaying RNA synthesis with [3H]-uridine is a more logical use of radioactive isotopes to study normal, nonproliferating corneal endothelial cell metabolism. But, radioisotopes have many disadvantages, including the terminal nature of the measurement, labor-intensive handling and disposal, and expense.

In this study, [3H]-thymidine could detect only the minimal endothelial cell DNA synthesis found at low, subconfluent cell numbers. Alamar blue is much more sensitive and specific as a measure of cell viability and does not depend on cell division. Because the corneal endothelial cell does not normally undergo mitosis, the Alamar blue assay is a more physiologic measure.

Fluorescence measurements of Alamar blue possess the advantage of having higher sensitivity to lower cell concentrations and minimal overlap of emission spectra, making percentage of reduction calculations unnecessary. We are currently investigating fluorescence data with this model.

The limitations of Alamar blue are few. As mentioned above, high cell concentrations and prolonged culture times will show reversal of the reduction process. Microbial contaminants will reduce Alamar blue and yield erroneous results, but contamination would adversely affect any assay of the cells. Bovine serum and phenol red may slightly interfere with the colorimetric changes. Although we have observed no sign of toxicity in our assays, long-term toxicity of Alamar blue to the endothelial cell was not ruled out by our results.

In conclusion, our results demonstrate that Alamar blue has several important advantages over other methods for assessing endothelial cells in vitro. Studies to develop Alamar blue-based determination of endothelial cell health in corneal organ cultures are under way.

Key Words
Alamar blue, corneal endothelium, in vitro assay, rabbit, viability

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References