The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells

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BACKGROUND: The current techniques for quantifying trophoblast viability, migration and invasion are mainly limited by the need to sacrifice the cells during the test procedure. In this study, the vital dye AB (AB) was used to quantify cell number and viability of BeWo and JEG-3 choriocarcinoma cells, as well as their migration and invasion through fibronectin-coated filters. METHODS: AB was directly added to culture medium of incubated test and control cells. At various time intervals, the redox reaction, in which AB is reduced by the cells, was measured by absorbance readings at 540 and 630 nm. For cell migration and invasion, cells were cultured onto uncoated or fibronectin-coated inserts, respectively. AB reduction of migrated cells was normalized to that of control cells to calculate percentages of migration. This model was also tested in the presence of a reported inhibitor, transforming growth factor (TGF) β. RESULTS: The curve of %AB reduction versus cell number was linear, with intra- and inter-assay Coefficient of Variations of 1.88% and 2.94%, respectively. AB reduction increased with both seeding concentrations and incubation time with AB. TGFβ treatment caused a modest decrease in AB reduction in both JEG-3 and BeWo cells. TGFβ treatment also decreased migration in BeWo, but not in JEG-3, cells. CONCLUSIONS: AB assay is a simple and reliable method for quantifying trophoblast viability, migration and invasion.

Key words: Alamar Blue/choriocarcinoma/invasion/migration/viability

Introduction

Trophoblast cell culture is a valuable tool in the research of placental physiology. Trophoblast cells, isolated from first trimester as well as term placentas, are utilized in different in vitro tests to study proliferation, invasion and differentiation. Quantitative assessment of these aspects relies on visual counting of entities of cells cultured in different conditions. The low reproducibility of this approach confers considerable limitations. Alternative approaches include spectrophotometric, fluorimetric and radiometric techniques to quantify appropriately labelled cells. These approaches have been used in the field of trophoblast research with variable success.

Alamar Blue (AB) is a water-soluble dye that has been previously used for quantifying in vitro viability of various cells (Fields and Lancaster, 1993; Ahmed et al., 1994). Due to the fact that it is extremely stable and more importantly nontoxic to the cells, continuous monitoring of cultures over time is possible (Ahmed et al., 1994). Mainly for this reason, this test has been considered superior to classical tests for cell viability such as the MTT test (Fields and Lancaster, 1993). The MTT test is based on the ability of viable cells to produce formazan from the cleavage of the tetrazolium salt by functional mitochondria (Mosmann, 1983). However, the MTT test necessitates killing the cells, making it impossible to follow-up cell cultures.

When added to cell cultures, the oxidized form of the AB enters the cytosol and is converted to the reduced form by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes. This redox reaction is accompanied by a shift in colour of the culture medium from indigo blue to fluorescent pink, which can be easily measured by colourimetric or fluorometric reading.

Choriocarcinoma cell lines have been useful as models for studying various functions of trophoblast cells including invasion and differentiation (King et al., 2000). We investigated whether AB can be used for quantifying not only cell number and viability, but also migration and invasion of JEG-3 and BeWo choriocarcinoma cells. The HT-1080 fibrosarcoma cell line was used as a control due to their high invasive capacity.
Here we describe the characteristics of this novel method in order to define the optimal culture conditions and to test the effects of transforming growth factor (TGF)β, a known inhibitor of migration.

Materials and methods

Materials

BeWo and JEG-3 choriocarcinoma cells were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and HT-1080 cells were a gift from the Centre of Human Genetics (UZ Gasthuisberg, Leuven, Belgium). AB was purchased from Biosource (Biosource Europa, Nivelles, Belgium), TGFβ from Sigma–Aldrich (Bornem, Belgium) and human fibronectin from DakoCytomation (Heverlee, Belgium). Materials for cell culture included 75 cm² Falcon flasks (BD Biosciences, Erembodegem, Belgium), 12 μm Millicel filters (Millipore, Amsterdam, The Netherlands) and 24-well Falcon plates (VWR International, Leuven, Belgium) which were used in the migration/invasion assays. Other products used in these experiments were Ham F12, Eagle’s minimum essential medium (EMEM), glutamine, heat inactivated fetal calf serum (FCS) (Sigma–Aldrich, Bornem, Belgium), Dulbeco’s modified Eagle’s medium (DMEM) (Gibco, Life Technologies, Belgium), penicillin–streptomycin solution, gentamicin solution, trypsin solution (Invitrogen life technologies, Merelbeke, Belgium), EDTA (Merck VWR International, Leuven, Belgium) and DNase I (Roche Diagnostics GmbH, Vilvoorde, Belgium).

Cell culture

Cells were maintained in 75 cm² Falcon culture flasks under standard culture conditions of 5% CO₂ in air at 37°C with medium renewal every 2–3 days. The culture media used were Ham F12 for BeWo, DMEM for HT-1080 and EMEM for JEG-3 cells, all containing 200 mM glutamine, 1/100 penicillin–streptomycin solution and 1/1000 gentamicin solution supplemented with 10% FCS. When confluent, cells were split 1 : 3. Cells passaged for 20, 11 and 15 times, for JEG-3, BeWo and HT-1080, respectively, were used for the experiments.

AB assay for cell quantification

Cells were trypsinized from subconfluent cultures by adding 3 ml of trypsin solution (100 mg EDTA, 100 mg DNase I, 5 ml penicillin–streptomycin, 500 μg gentamicin in 500 ml trypsin) to 50 ml Falcon flasks with confluent cells followed by 10 min incubation at 37°C with regular gentle shaking. The trypsin reaction was stopped by adding 10 ml of appropriate culture medium containing 10% FCS. The cell suspension was then centrifuged at 750g for 10 min at 20°C. The cell pellet was suspended in 2 ml of medium with 1% FCS and thoroughly mixed by repeated pipetting. Cells were then counted with a Fuchs–Rosenthal hemacytometer and brought first to a concentration of 1 × 10⁶ cells/ml and subsequently to serial 1 : 2 dilutions. The resulting different cell suspensions were seeded into duplicate wells of a 96-well plate (200 μl well⁻¹) and incubated at 37°C. After an initial 4 h period to allow cell attachment, AB solution was directly added to the medium resulting in a final concentration of 10%. As negative control AB was added into the medium without cells. The plate was further incubated for 24 h at 37°C. The absorbance of test and control wells was read at 540 and 630 nm with a standard spectrophotometer.

The number of viable cells correlates with the magnitude of dye reduction and is expressed as percentage of AB reduction (Ahmed et al., 1994; Goegang et al., 1995; Nociari et al., 1998). The calculation of the percentage of AB reduction (%AB reduction) is as follows according to the manufacturer’s protocol:

\[
\%AB reduction = \frac{(e_{ox}A_2)(A_1)}{(e_{red}A_0)(A_2)} - \frac{(e_{ox}A_0)(A_1)}{(e_{red}A_0)(A_2)} \times 100
\]

In the formula, \(e_1\) and \(e_2\) are constants representing the molar extinction coefficient of AB at 540 and 630 nm, respectively, in the oxidized (\(e_{ox}\)) and reduced (\(e_{red}\)) forms. \(A_1\) and \(A_2\) represent absorbance of test wells at 540 and 630 nm, respectively. \(A'_1\) and \(A'_2\) represent absorbance of negative control wells at 540 and 630 nm, respectively. The values of %AB reduction were corrected for background values of negative controls containing medium without cells.

In order to eliminate differences due to medium colour, the experiment was repeated using culture media filtered with sterile activated charcoal to remove phenol red from culture media. Brieﬂy, after adding heat inactivated FCS to the medium, 16.5 mg activated charcoal was added per 1 ml medium, the bottle was thoroughly shaken for 30 min, centrifuged at 3000 RPM for 10 min and the supernatant was filtered to yield a colourless medium.

AB assay for cell viability/proliferation

Initial experiments were carried out to follow %AB reduction over time. The aim was to determine the optimal seeding density and culture period. JEG-3, HT-1080 and BeWo cells were trypsinized from subconfluent cultures as described earlier, were suspended in culture medium containing 10% FCS, and then seeded into duplicate wells of a 96-well plate (200 μl well⁻¹) at concentrations of 1.5 × 10⁶–1 × 10⁷ cells/ml at standard culture conditions of 5% CO₂ in air at 37°C. After an initial 4 h period to allow for cell attachment, AB was added directly into culture medium at a final concentration of 10% and the plate was returned to the incubator. Optical density of the plate was measured at 540 and 630 nm with a standard spectrophotometer at 1, 2.5, 4, 24, 48, 72 and 168 h after adding AB. Because the culture medium was not changed during this period, the calculated %AB reduction is a cumulative value. As a negative control, AB was added to medium without cells.

Next, the viability of JEG-3, HT-1080 and BeWo cells was tested in the presence of a reported inhibitor, TGFβ. Cells were seeded into 96-well plates at a concentration of 1 × 10⁶ cells/ml for 24 h in a standard incubator. After 24 h, cells received fresh medium with or without 5 ng TGFβ. After another 24 h of culture, AB was added directly into culture media at a final concentration of 10% and optical densities were measured after yet another 24 h period.

AB assay for cell migration/invasion

A 24-well Falcon plate was used as a ‘feeder tray’ in which 600 μl of culture medium was placed in each well. For the migration assay, we used 12 μm Millicel filters, with a 12 micro-pore polycarbonate membrane. For the invasion assay, the same filters were pre-coated with fibronectin. Briefly, 200 μl of 20 μg ml⁻¹ fibronectin solution was added on top of the filters and incubated for 30 min at 37°C, followed by removal of the excess fibronectin solution. In both migration and invasion tests, 400 μl of cell suspension (at 1 × 10⁶ cells/ml) was seeded on top of the filters which were then immersed into the feeder trays (Figure 1A) and incubated at 37°C in standard conditions. After 4 h of initial cell attachment, the medium in the feeder tray was changed into either control medium or medium containing 5 ng of TGFβ. An incubation period of 24 h was allowed for cells to invade
the fibronectin layer and migrate through the filter pores to become ultimately attached to the lower side of the filter immersed in the feeder tray. Cells on top of the filters were then wiped away with a sterile cotton swab, AB was added to the medium of the feeder tray to make a final concentration of 10% and the plate was further incubated at 37°C. After 24 h of incubation with AB solution, 200 μl of conditioned culture medium was transferred into a new 96-well plate. The absorbance of test and control wells was read at 540 and 630 nm with a standard spectrophotometer. Figure 1B shows colour changes in different wells representing cells cultured above filters with or without TGFβ treatment, wells with positive controls (cells without filters); c, negative controls (medium only); d, cells after invasion + TGFβ; e, cells after invasion (no TGFβ).

Figure 1. (A) The invasion assay. Cells were cultured on top of fibronectin-coated Millicel filters immersed into wells of a feeder tray containing culture medium (+ FCS 1%). After 24 h of incubation, Almar Blue (AB) was added to the medium of the feeder tray and became gradually reduced by cells which had invaded the fibronectin layer and migrated through the filter pores, causing a shift in colour from blue to pink. (B) Changes of medium colour associated with AB reduction from blue (oxidized) to pink (reduced) in duplicate wells. a, positive control (cells + TGFβ without filters); b, positive controls (cells without filters); c, negative controls (medium only); d, cells after invasion + TGFβ; e, cells after invasion (no TGFβ).

The AB reduction in the conditioned medium was calculated as described earlier and the %AB reduction correlated with the number of migrated (or invaded) cells present on the lower side of the filters. The %AB reduction of migrated or invaded cells was normalized to %AB reduction of an equal number of cells cultured in the same conditions but without filter. Thus, the percentage of migrated (%MIG) or invaded (%INV) cells was calculated as follows:

\[
\%\text{MIG(or INV)} = \frac{\%\text{AB reduction of migrated (or invaded) cells}}{\%\text{AB reduction of cells without filters}} \times 100\%
\]

Statistical analysis
Statistical analysis was carried out using GraphPad Prism software, version 3.0. A P-value of <0.05 was considered significant. For intra- and inter-assay variability, data from three consecutive experiments were analysed. A standard curve of %AB reduction was plotted and linear regression analysis was carried out to calculate \(r^2\) and P-values for the slope. The Mann–Whitney test was used to compare viability and percentage of migrated (%MIG) and invaded (%INV) cells between control and TGFβ-treated cells.

Results

**AB reduction for cell number quantification**
When %AB reduction was plotted against the logarithm of cell concentrations, the standard curve was linear between 16.6 × 10³ and 500 × 10³ cells/ml, with \(r^2\) values of 0.73, 0.81 and 0.81 and P-values for the slope of 0.014, 0.005 and 0.006, for HT-1080, JEG-3 and BeWo cells, respectively (Figure 2A). The standard curves showed a shift in intercept with better linearity for HT-1080 and JEG-3 cells (B). Data are presented as means ± SD.
following experiments were all carried out with charcoal-filtered media.

**AB assay for cell viability: effect of incubation time, cell density and TGFβ treatment**

To determine the optimal working cell density and incubation time, the %AB reduction was followed in different cell concentrations up to 168 h in culture. Cell viability at each time point was confirmed by direct microscopy and by performing an MTT test (results not shown). The %AB reduction increased over culture time, from a starting cell density of $50 \times 10^3$ cells/ml and higher, and starting from 4 h after addition of AB (Figure 3). Because culture medium was not changed during the incubation period and because cell proliferation continues during this period, the measurements at each time point represent the cumulative %AB reduction. On the basis of these results and the findings of other groups (Nociari et al., 1998; O’Brien et al., 2000), we decided for the following experiments to use a cell concentration of $1 \times 10^5$ cells/ml and an incubation time with AB of 24 h.

The viability of JEG-3 and BeWo cells was tested in the presence of a reported inhibitor, TGFβ. Addition of 5 ng TGFβ decreased %AB reduction in cultures of both JEG-3 (67.87 versus 71.71) ($P < 0.05$, $n = 5$) and BeWo cells (55.88 versus 71.43) ($P < 0.01$, $n = 3$) after 24 h of culture. TGFβ had no effect on %AB reduction in HT-1080 cells.

**AB assay for cell migration and invasion**

Cell migration and invasion was examined after culturing cells on top of uncoated and fibronectin-coated filters, respectively. Migration of cells through filters was confirmed by visualizing the migrated cells with an inverted microscope on the bottom of the filter after incubation with AB and after crystal violet staining. The intra-assay variability for %MIG and %INV of the filter after incubation with AB and after crystal violet lyses to be carried out on the same sample including histology and flow cytometry. One of those vital dyes is the recently introduced AB, which is reduced to a fluorescent pink dye by

Discussion

Although several tests are available for determining cell number and viability as well as assessing migration and invasion of trophoblasts, their applicability is limited by the need to sacrifice the cells during the test procedure. To tackle this problem, vital dyes which are non-toxic to cells have been introduced. They offer the advantages of keeping the cells in culture to observe changes over time and of allowing other analyses to be carried out on the same sample including histology and flow cytometry. One of those vital dyes is the recently introduced AB, which is reduced to a fluorescent pink dye by
the metabolic activity of living cells, ranging from bacteria to mammalian cells. AB is considered superior to other cell viability assays, such as the MTT test, because it is not toxic to cells and does not necessitate killing them during the assay procedure (Ahmed et al., 1994). Moreover, AB can also be reduced by cytochromes, whereas tetrazolium salts such as MTT are not. This might explain the higher sensitivity and reproducibility of AB in detecting low cell concentrations (Nociari et al., 1998).

AB has been utilized in various tests, e.g. for measuring the proliferation of human lymphocytes (Ahmed et al., 1994; de Fries and Mitsuhashi, 1995), primary rat hepatocytes (O’Brien et al., 2000) and various human cell lines (Fields and Lancaster, 1993), as well as for measuring cell-mediated cytotoxicity of human T lymphocytes (Nociari et al., 1998) and murine macrophages (Ashany et al., 1995).

So far, there has been no report on the use of AB in ectodermal cell lines. In this study, we report on the applicability of AB assay for quantifying cell numbers and viability of BeWo and JEG-3 choriocarcinoma cells. In addition, we applied this method to quantify migration and invasion into fibronectin-coated filters. As a positive control, we used the fibrosarcoma cell line HT-1080, a mesothelial cell model with well-known invasive properties. Due to the homogeneity of choriocarcinoma cell lines and the relatively simple propagation procedures, they were preferred in this study to primary trophoblast cultures (King et al., 2000). Moreover, trophoblast cultures from term placentas show relatively low proliferative and invasive capacities compared with their malignant counterparts making them less attractive models for the study of cell migration and invasion.

**Standard curve of AB reduction**

AB reduction showed high reproducibility with very low intra-assay and inter-assay CV, comparable with the MTT test. There was also a good linear correlation between %AB reduction and cell concentrations, over a range of $16.6 \times 10^3$ to $500 \times 10^3$ cells/ml, with $r^2$ values of 0.73, 0.81 and 0.81 for HT-1080, JEG-3 and BeWo cells, respectively. Similar findings have been obtained by other groups working with AB (Ahmed et al., 1994; Goegan et al., 1995; Nociari et al., 1998; O’Brien et al., 2000).

The initial experiments were carried out with coloured media. Interference from medium colour was excluded by removing the indicator Phenol Red by charcoal treatment. Thereafter, the standard curves of %AB reduction versus cell number showed a shift in intercept. Additionally, differences in %AB reduction were found between the same concentrations of BeWo, JEG-3 and HT cells. This can be attributed to different rates of metabolic activity, proliferation and differentiation (Sullivan, 2004; Al-Nasiry et al., 2006). Furthermore, it has been shown that proteins in culture media can cause spectral shifts and may affect the kinetics of the assay e.g. the rate of uptake of substrate by the cells (Googan et al., 1995). Therefore, caution is needed when comparing %AB reduction of different cell types, and errors may arise when extrapolating the curves obtained by one cell type to another.

**Characteristics of viability/proliferation model**

Addition of AB to cultured cells does not alter their viability, unlike that which occurs during monitoring by Trypan Blue exclusion (Ahmed et al., 1994; Nociari et al., 1998). We confirmed the viability of the cells subjected to AB by direct microscopy and by performing an MTT test on cells after treatment with AB. A cumulative increase in AB reduction could be found when various concentrations of cells were followed-up over 7 days in culture, reflecting ongoing cell proliferation. This raises the need to standardize seeding density and incubation time with AB when comparing different culture conditions.

TGFβ has been reported to decrease proliferation of a first trimester trophoblast cell line HTR-8. No such effects were seen in either JEG-3 and JAR choriocarcinoma lines (Graham et al., 1994) or cultured first trimester trophoblasts (Graham and Lala, 1991) using the [3H] Thymidine incorporation test. We found in TGFβ-treated JEG-3 cells, a decreased %AB reduction compared with control cells suggesting a certain degree of inhibitory response in these cells. This contradictory finding could be partly explained by the higher sensitivity of the AB assay than [3H] Thymidine incorporation test in detecting modest differences in cell number.

**Characteristics of the migration/invasion model**

Preliminary trials with a commercially available invasion kit (QCM 555, Chemicon) were hampered by the high intra-assay variability and the need for labelling cells with fluorescent markers (S. Al-Nasiry, unpublished observations). In our model, cell migration was achieved by culturing the cells on top of 12 μm Millicell filters with a 12 micro-pores polycarbonate membrane, through which cells are allowed to migrate for 24 h. For the cell invasion experiments, we used an identical setting with the additional step of coating the filters with human fibronectin solution. We confirmed the presence of cells on the bottom side of the filter by direct visualization after crystal violet staining in both settings.

To calculate the %MIG or %INV, we related the %AB reduction to that of control wells without inserts containing the same concentration of cells under the same conditions. Hundred percentage AB reduction of these control wells represents the value expected if all cells within the inserts would have migrated through the filters and have reduced AB in the feeder tray. This ‘normalized value’ takes into account the continuous proliferation of the cells. Relating the %AB reduction to that of cells before migration or invasion would lead to overestimation of %MIG or %INV due to the possible interference of proliferation.

The intra-assay variability for %MIG and %INV was 18% and 6.7%, respectively, which is satisfactory, considering the inherent variability of the technique itself and the biological variability of cells used. With increasing cell density, there was a trend for a higher %MIG as well as higher %INV, although the latter was statistically not significant. This observation excludes a saturation effect with higher cell densities. On the basis of these findings and those of other groups (Nociari et al., 1998), we used a concentration of $1 \times 10^5$...
Cells cultured on fibronectin-coated filters had consistently higher %AB reduction when compared with cells cultured on uncoated filters (data not shown). It is indeed well known that fibronectin enhances attachment and viability of term trophoblasts (Pijnenborg et al., 2000), as well as choriocarcinoma cells (Al-Nasiry et al., 2003). Stimulation of proliferation and/or migration by interaction with fibronectin is another possible explanation (Armant, 2005).

Although TGFβ is reported to inhibit trophoblast migration by different mechanisms including increasing the adhesiveness to ECM (Irving and Lala, 1995) and up-regulating TIMP’s (Graham and Lala, 1991), JEG-3 and JAR cell lines appear to be resistant to these effects (Graham et al., 1994). This resistance could be explained by the loss of smad3 transcription factors (Xu et al., 2002). However, our results on migration suggest that BeWo and, to a lesser degree, JEG-3 cells show some inhibitory response to TGFβ. This might be explained by the different type of inserts and ECM used in our model (fibronectin versus Matrigel), or again by the high sensitivity of the AB assay in detecting subtle differences in the number of migrated cells.

In conclusion, this study endorses the use of AB assay as a reliable method of assessing the viability, migration and invasion of choriocarcinoma cells which can be used as a model of invasive trophoblasts. Such models may prove valuable in our continuing search for in vitro assays which mimic the in vivo environment of the trophoblast.

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