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Human glioma demonstrates cell line specific results with ATP-based chemiluminescent cellular proliferation assays

Michael E. Sughrue, Martin J. Rutkowski, Ari J. Kane, and Andrew T. Parsa*

Department of Neurological Surgery, University of California at San Francisco, 505 Parnassus Avenue, San Francisco, California 94143, USA

Abstract

Alteration of tumor cell growth kinetics is the goal of nearly all current or proposed therapies for human neoplasms. The adenosine triphosphate (ATP) chemiluminescent assay has been used for some time as a surrogate marker of *in vitro* cell growth. Here we present data showing that three human glioblastoma cell lines (U87, U251, G55) demonstrate significantly different cell number to luminescence relationships when subjected to this assay. We plated progressively increasing numbers of cells per well; from 1000 to 50,000 were grown in Dulbecco's modified Eagle's medium without serum and cultured for 6 hours. Cells were then lysed and subjected to the chemiluminescent assay to measure ATP levels and a linear relationship between cell number and measured luminescence was found. Despite this, we found that the slope of the regression line () varied markedly between different cell lines (U251 [$= 0.968 \pm 0.3$] vs. U87 [$= 0.772 \pm 0.2$] vs. G55 [$= 0.757 \pm 0.2$]; $p < 0.0001$), suggesting a difference in ATP luminescence per cell between these cell lines. Thus, we have demonstrated that luminescence values are internally linear within a given cell population, but luminescence level per cell varies significantly between different glioma cell lines. Our findings suggest that different glioma cell lines have unique levels of ATP per cell.

Keywords

ATP assay; Cell division; Chemiluminescence; Chemotherapy; Glioma; Proliferation

1. Introduction

Alteration of tumor cell growth kinetics is the goal of nearly all current or proposed therapies for human neoplasms.^{1,2} It follows that quantitative measurement of tumor cell kinetics represents an important endpoint for evaluation of putative anti-cancer therapies. *In vivo* experimental neoplasms usually form a focal mass, the kinetics of which can be quantified by measuring its size or its clinical effects on the host animal (that is, overall survival and symptom progression). Determining the kinetics of *in vitro* growth of neoplastic cells, however, is less observational and typically requires the measurement of a molecular surrogate of growth.^{1–3}

One surrogate marker of *in vitro* cell growth used to quantify cell growth for some time is the adenosine triphosphate (ATP) chemiluminescent assay.^{4–11} Total ATP is quantified from cell lysates using a chemiluminescent luciferase reporter assay. Because ATP is present in viable cells but rapidly depleted in dead cells, total ATP is expected to rise in a linear

fashion directly proportional to the number of cells present. This assay has the distinct advantage of being very rapid compared to many other *in vitro* cell proliferation assays, and validity of the hypothesis that ATP levels in the cellular lysates are directly proportional to cell number has been demonstrated repeatedly.^{9–11}

Given the simplicity and reliability of the ATP chemiluminescent assay, it is an ideal assay for high throughput screening of tumor cells for sensitivity to cytotoxic/cytostatic agents.^{1–3,10–15} For this reason, this assay has been proposed as the cornerstone of a multi-agent sensitivity screen aimed at patient-specific optimization of chemotherapeutic regimens for ovarian cancer.^{1–3,10–15} The application of this somewhat non-specific assay to a potentially infinite number of cell types seen in human tumors is appealing, but raises significant issues regarding data analysis. More specifically, while ATP levels rise in a linear fashion with increasing cell number in a given homogeneous cell population, it is possible that the metabolic machinery and overall ATP level varies between cell types. If true, this lack of consistency between different cell populations could lead to different levels of luminescence for the same number of cells, making it difficult to compare growth rates among different cell lines exposed to the same conditions. This problem becomes acutely problematic when working with *ex vivo* samples from individual patients' tumor specimens,^{1–3,10–15} which are almost certainly polyclonal cell populations.¹⁶ Here we present data demonstrating that subjecting different glioma cell lines to an ATP chemiluminescence cell proliferation assay yields significantly different cell to luminescence relationships.

2. Methods and materials

2.1. Cell lines and cell culture techniques

Three human glioblastoma cell lines (U87, U251, G55) were used. The U87 and U251 cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). G55 is a human glioblastoma cell line that has been passaged through nude mice and re-established as a stable xenograft cell line. The G55 cell line was kindly donated by C. David James (Department of Neurological Surgery, University of California at San Francisco). In addition, human cervical cancer (HeLa), and chondrosarcoma cell lines were purchased from ATCC and subjected to the same analyses to provide other cancer cells lines for comparison.

Cell lines were maintained at 37 °C, 95% oxygen gas, and 5% carbon dioxide in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) without phenol red, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 5% penicillin–streptomycin (100 units/mL penicillin, and 100 µg/mL streptomycin). Cells that had undergone five or fewer passages after thawing were selected; all had been passed many times before we established these lines. Cell lines were cultured, grown until approximately 80% confluent, washed in 1% Dulbecco's phosphate buffered saline (pH 7.4), then trypsinized. Cells were then centrifuged, resuspended in serum-free and antibiotic-free DMEM, and then counted using a hemocytometer. To minimize sampling bias, cell concentrations were determined by performing five separate measurements and using their mean. Cells were then plated in a 96-well plate with serum-free and antibiotic-free DMEM at progressively increasing cell numbers, from 1000 to 50,000 cells per well. Each cell number was plated eight times. Cells were plated for 6 hours to allow adequate time for adherence, but minimal time for division. Examples of the microscopic appearance of each cell line plated at 500, 5000, 10,000, and 50,000 cell density in the 96-well plates are provided in Fig. 1.

2.2. Baseline ATP chemiluminescence and MTT assays

ATP chemiluminescence was determined using the ATPlite 1Step Luminescence Assay System® (PerkinElmer; Waltham, MA, USA) as per the manufacturer's protocol. Each well was cleared of DMEM, cells were exposed to lysis buffer and luciferase/D-luciferin substrate, and then mixed on a plate shaker for 5 minutes in the dark. Luminescence was determined using a spectrophotometer (Biotek Synergy 2; BioTek Instruments; Winooski, VT, USA) with a 1 s exposure time. Data were analyzed using Biotek Generation 5 software.

As a confirmatory test, we also repeated the same protocol using the thiazoyl blue; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based cell proliferation assay (Invitrogen), which was performed according the manufacturer's instructions. In short, after allowing cells to adhere, we removed the medium and replaced it with 100 μ L of fresh culture medium (DMEM without phenol red), with 10 μ L of the 12 mM MTT stock to each well. Cells were incubated in MTT at 37 °C for 4 hours. At this point all but 25 μ L of medium was removed from the wells, 50 μ L of DMSO were added to each well, the wells were mixed thoroughly, and incubated at 37 °C for 10 minutes. Absorbance was then analyzed at 540 nm.

2.3. Statistical analysis

The relationship between cell number and luminescence was studied using linear regression, and expressed as the r -value (slope) and y-intercept. The p value of the regression equation was considered significant below the 5% level (i.e. $p < 0.05$).

3. Results

3.1. Reliability of cell plating protocol

Our method of cell number analysis using the ATP-based chemiluminescence assay yielded a linear relationship between intended cell number and measured luminescence. Further, the inter-observational variation for different intended cell numbers was rather small (Fig. 2). Together, these observations suggest that our method for plating cells successfully maintained a consistent number of cells per well among our repetitions. Adequate time for adherence minimized both loss of cells and cellular division, resulting in minimal alterations to our intended number of cells per well; at the very least, any systematic error that may have been present in measured cell number was consistent and varied in a linear fashion among the different cell lines.

3.2. ATP chemiluminescence per cell in three glioma cell lines

We found marked differences in ATP chemiluminescence per cell among the three different glioma cell lines U251, U87 and G55. Regression analysis demonstrated a linear relationship between intended cell number and chemiluminescence in all instances ($p < 0.00001$) (Table 1). Despite a strong linear correlation between plated cell number and luminescence, we found that the slope of the regression line varied markedly between the three different cell lines (U251 [$r = 0.90 \pm 0.001$] vs. U87 [$r = 0.032 \pm 0.001$] vs. G55 [$r = 0.62 \pm 0.001$]; analysis of variance $p < 0.0001$) (Fig. 2a, c, e), suggesting a difference in the ATP chemiluminescence per cell among these cell lines. In contrast, the MTT assay provided essentially identical cell number to luminescence relationships (reflected by similar r values and variances between cell lines), suggesting that these between-cell-line differences represent a feature of the ATP assay, and are not due to methodologic error in cell plating.

Similar assays were performed on HeLa and the chondrosarcoma cell line SW1353, which demonstrated that while between-cell line ATP assay variability is a feature of glioma cells, it is a feature of other cancer cell lines as well (HeLa [$\sigma = 0.36 \pm 0.001$] and SW1353 [$\sigma = 0.16 \pm 0.002$]).

4. Discussion

The ATP chemiluminescence assay is a well established method of quantifying cell proliferation and studying the effects of cytotoxic and cytostatic agents on eukaryotic cells in vitro.^{1–3,10–15} In this study, we demonstrate that luminescence values are directly proportional to cell number, rising in a linear relationship with increasing cell number.¹⁷ However, these linear relationships are not entirely consistent between different cell lines or different cancer types. The linear regression performed demonstrates that the luminescence values for the ATP assay are very highly linear across a range of cell numbers, and that the linear relationship differs in slope and y-intercept between different cell lines, suggesting that within an appropriate range of cell number, the luminescence per cell is different between different cell lines. While the curves approach each other at low cell values and appear more similar at these lower values, this is because the difference is larger at higher cell numbers, and the values are plotted on an axis designed for these large values. If plotted on a smaller y-axis, these values will appear more different.

As demonstrated by our study and the published literature, a large number of trials can be performed rapidly and reliably,^{2,7,18} which is a major factor favoring the use of this assay. Further, despite this variability, our data suggest that the ATP assay provides more precise estimates of cell number with less variability than the MTT assay, which is another plus. For these reasons, patient-specific screening of the effect of various agents on tumor cells using this assay has been proposed as one method for determining chemoresistance of tumors prior to administration of potentially toxic therapies.^{1–3,10–15} Additionally, some groups have proposed the use of this assay as a surrogate for cytokine function.³ While evidence-based, patient-directed chemotherapy represents a desirable goal, significant questions remain regarding the use of this approach in clinical practice. Most notably, this assay utilizes a relatively non-specific molecular marker (ATP) as a surrogate marker of cell number.^{4–7,9,18,19} While data suggest that this relationship remains reliable for comparisons within a monoclonal population of cells, a clinically viable susceptibility assay must be applicable to the mixed cell populations seen in human tumors.¹⁶ Furthermore, for any such assay to gain significant acceptance in clinical oncology, some method of comparison of results between patients must be possible. This would enable decision making on whether or not to administer a given agent, and would allow the reliability of these assays to be validated across large cohorts of patients.¹⁵

Although we did not address the specific cause of this correlation, our results suggest that glioma cell lines U251, U87, and G55 likely contain different levels of ATP per cell. Differences in ATP production have previously been demonstrated to exist among different tumor cell types and can be modulated by factors such as changes in levels of glycolytic or mitochondrial ATP production.^{20–22} In light of these issues, our results raise the possibility that the absolute reduction of ATP chemiluminescence by a given cytotoxic agent may not necessarily predict clinical success with this agent, as an identical absolute reduction in chemiluminescence could represent a different absolute number of cells killed for different neoplastic cell populations. One approach to addressing this interpopulation variability is the estimation of ATP chemiluminescence per cell as a preliminary step towards normalizing patient data onto a comparable scale. Although this would not necessarily eliminate issues with comparison of heterogeneous cell populations, it might limit the impact of this problem. The possibility that a specific chemotherapeutic agent could select for a specific

cell population with different ATP levels than the parent cell population might represent the limits of this assay's clinical potential. Ultimately, our results call attention to the heterogeneity of human neoplastic cells in purportedly similar tumor types, both in terms of likely differences in ATP metabolism and the potential for clones to divide at significantly different rates.

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Fig. 1.

Black-and-white brightfield images of three human glioblastoma cell lines (U87, U251, G55) showing the morphologic appearance of: (a–d) adherent U251; (e–h) U87; and (i–l) G55 cells plated in a 96-well plate at (a, e, i) 500 cells; (b, f, j) 5,000 cells; (c, g, k) 10,000 cells; and (d, h, l) 50,000 cells per well (magnification, $\times 10$). Images were acquired immediately prior to analysis.

Fig. 2.

Regression plots demonstrating the relationship between cell number *versus* luminescence for: (a, c, e, g, i) the adenosine triphosphate (ATP) chemiluminescent assay; and (b, d, f, h, j) MTT assays performed on the (a, b) U251, (c, d) U87, (e, f) G55 (g, h) human cervical cancer (HeLa), and (i, j) SW1353 cell lines. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 1

Slope, y-intercept, and variances for the linear regressions of cell number *versus* luminescence for all five cell lines analyzed with the adenosine triphosphate (ATP) chemiluminescent and MTT assays

Assay and cell line ^a	SE	y-Int	SE	p value	SE/ (%)
<i>ATP</i>					
U251	0.090	0.001	126	19	<0.00001 1.1
U87	0.032	0.001	270	15	<0.00001 3.1
G55	0.062	0.001	140	20	<0.00001 1.6
HeLa	0.036	0.001	94	19	<0.00001 0.3
SW1353	0.160	0.002	452	32	<0.00001 1.3
<i>MTT</i>					
U251	4×10^{-6}	4×10^{-7}	0.10	0.01	<0.00001 10.0
U87	4×10^{-6}	3×10^{-7}	0.06	0.01	<0.00001 7.5
G55	4×10^{-6}	5×10^{-7}	0.06	0.01	<0.00001 12.5
HeLa	8×10^{-6}	3×10^{-7}	0.10	0.01	<0.00001 3.8
SW1353	3×10^{-6}	1×10^{-7}	0.01	0.002	<0.00001 3.3

= slope of the linear regression, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SE = standard error, y-Int = y intercept.

^aGlioma cell lines = U251, U87, G55; other cell lines = HeLa and SW1353.