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Laboratory Study

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

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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 (β) varied markedly between different cell lines (U251 [$\beta = 0.968 \pm 0.3$] *vs.* U87 [$\beta = 0.772 \pm 0.2$] *vs.* G55 [$\beta = 0.757 \pm 0.2$]; $p < 0.0001$), 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.
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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.

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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

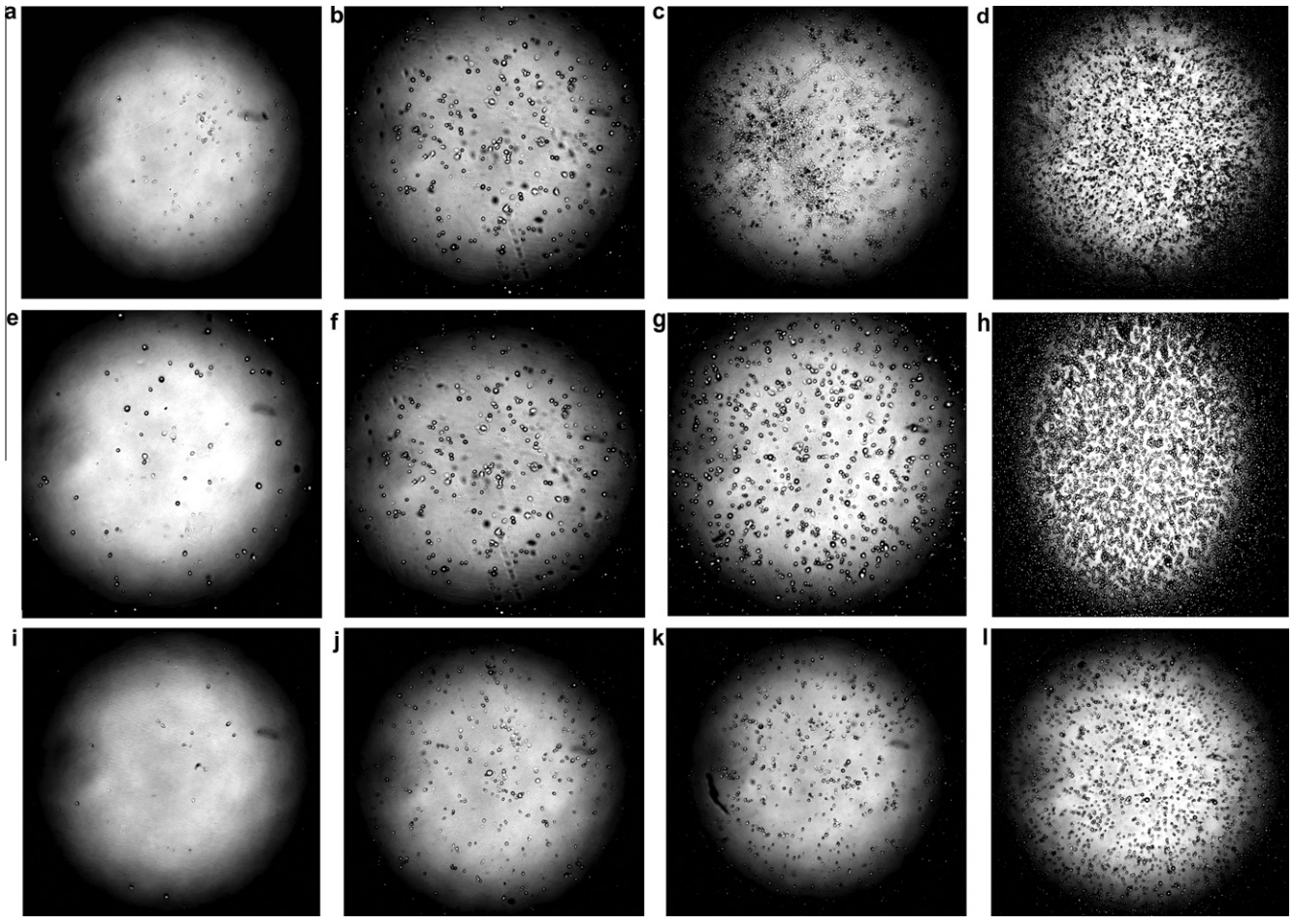

Fig. 1. Black-and-white brightfield images of three human glioblastoma cell lines (U87, U251, G55) showing the morphologic appearance of: (a–d) adherent U251; (e–h) U87; and (i–l) G55 cells plated in a 96-well plate at (a, e, i) 500 cells; (b, f, j) 5,000 cells; (c, g, k) 10,000 cells; and (d, h, l) 50,000 cells per well (magnification, ×10). Images were acquired immediately prior to analysis.

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