Identification of Anti-Invasive but Noncytotoxic Chemotherapeutic Agents Using the Tetrazolium Dye MTT to Quantitate Viable Cells in Matrigel

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ABSTRACT

Screening methods for chemotherapeutic agents usually rely on the cytotoxic properties of the drugs. However, agents that inhibit invasion may have more efficacy and cause fewer side effects. Various cellular invasion assays have been used to evaluate these types of compounds, including the modified Boyden chamber, monolayer wound models and Matrigel outgrowth assays. In this report, we have combined the use of the Matrigel outgrowth assay with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) visualization and cell viability dye to visualize invasive cells on Matrigel without magnification. Extraction of the dye's formazan byproduct allows cell viability to be assessed. Using several invasive and noninvasive cell lines, the utility of the method for various target cells was verified. Several established chemotherapeutic agents were also screened for their anti-invasive and/or cytotoxic effects when cultured on Matrigel. Our results suggest that this method may be an easy, inexpensive and nonradioactive alternative for both enumerating cells on Matrigel and screening various tumor cell lines treated with chemotherapeutic agent to look for compounds with noncytotoxic but anti-invasive properties.

INTRODUCTION

Cancer treatment to eliminate cancer cells and prevent tumor metastasis is often limited because toxic chemotherapeutic agents affect both rapidly dividing normal cells and cancer cells. Recent studies have focused on the development of nontoxic pharmacological agents that inhibit metastasis or invasion. Various effective methods to screen numerous compounds for cytotoxic activity have been developed, but the same is not true for anti-invasive drugs. The modified Boyden chamber is one of the classic in vitro methods to assess tumor cell invasion (1). Nevertheless, the use of this method to screen potential drugs is costly (both chamber and visualization method) and labor-intensive. Similarly, an animal model to screen anti-invasive drugs would be too expensive and time-consuming.

Matrigel is a biological extracellular matrix extracted from the Englebreth-Holm-Swarm (EHS) mouse tumor consisting of the major basement membrane components laminin, entactin, collagen IV and heparan sulfate proteoglycan (5). Matrigel at room temperature forms a gel that can support the growth of primary and continuous animal adherent cell lines. Numerous studies have indicated that Matrigel has the biological properties of an in vitro reconstituted basement membrane functioning as a medium for cell attachment, growth, differentiation, migration and invasion (2). In addition, Matrigel acts as a reservoir for growth factors, angiogenic factors and proteinases. Previous methods to assess cell viablity on Matrigel have consisted of either [^{3}H]thymidine uptake or enumeration of cells after trypan blue staining. Although [^{3}H]thymidine uptake is sensitive and allows the detection of DNA synthesis, it does not indicate cell viability or proliferation. Extraction of DNA from Matrigel, loss of cell morphology and generation of radioactive waste are also some of the obstacles with the method. Likewise, removal of viable cells from Matrigel with proteinases (e.g., trypsin or Dispase) is difficult and does not remove cells that have invaded the Matrigel.

We describe a method of quantitating viable cells in Matrigel using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (4). MTT is cleaved by a mitochondrial hydrolase in metabolically active cells to yield a formazan dye that is detectable by spectrophotometry. Because the dye is converted in situ inside viable cells, the method also allows for macroscopic visualization of invasive cellular networks on Matrigel. By combining the use of MTT to quantitate viable cells and Matrigel basement membrane to assess the capacity for cell invasion, this method can be used to screen potential drugs for both anti-proliferative (or cytotoxic) and anti-invasive properties without the use of a microscope. Using this assay, various cancer cell lines were screened for their invasive properties, and the viability of these cells was determined. Several established chemotherapeutic drugs were tested for cytotoxic and anti-invasive activities. Furthermore, compounds that interfere with cell to extracellular

matrix adhesion could be screened with this method. It is also possible to apply this method to screen compounds in other biological systems. For example, because endothelial cells differentiate on Matrigel and form cellular networks that mimic angiogenesis, this method could be used to screen compounds that either facilitate or inhibit angiogenesis. Other cells that can be studied with this method include macrophages, vascular smooth-muscle cells and mammary epithelial cells. Thus, depending on the biological system used, the Matrigel-MTT assay can potentially screen various compounds that affect cellular behavior but not viability. The method could be implemented at research sites that screen a variety of natural compounds for anti-invasive properties and by pharmaceutical companies to screen various drugs for anti-invasive but non-cytotoxic activity.

MATERIALS AND METHODS

Reagents and Cell Lines

Noninvasive MCF-7 and RKO.E6 cell lines and invasive MDA.MB.231 and A549 cells were obtained from ATCC (Rockville, MD, USA). Invasive prostate Tsu-pr1 cells were from laboratory stocks (7). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin and cultured in 5% CO_2 at 37°C. The chemotherapeutic agents doxorubicin, etoposide, suramin, taxol and minocycline were obtained from Sigma Chemical (St. Louis, MO, USA).

MTT Viability Assay

Viable cells were detected by measuring the conversion of the tetrazolium dye MTT (Sigma Chemical) to formazan as described by Mossman (6) with some modification. Different numbers of cells, as described in the figures, were plated in a 24-well uncoated tissue culture dish or in a tissue culture dish coated with 0.3 mL Matrigel as described below. MTT (0.5 mg/mL in phosphate-buffered saline) was added to each well and incubated for 4 h. The crystallized formazan dye was solubilized after adding detergent (5% sodium dodecyl sulfate [SDS] and 0.05 *N* HCl). Absorbance was measured at 595 nm with a Model 5550 Microplate Reader (Bio-Rad, Hercules,

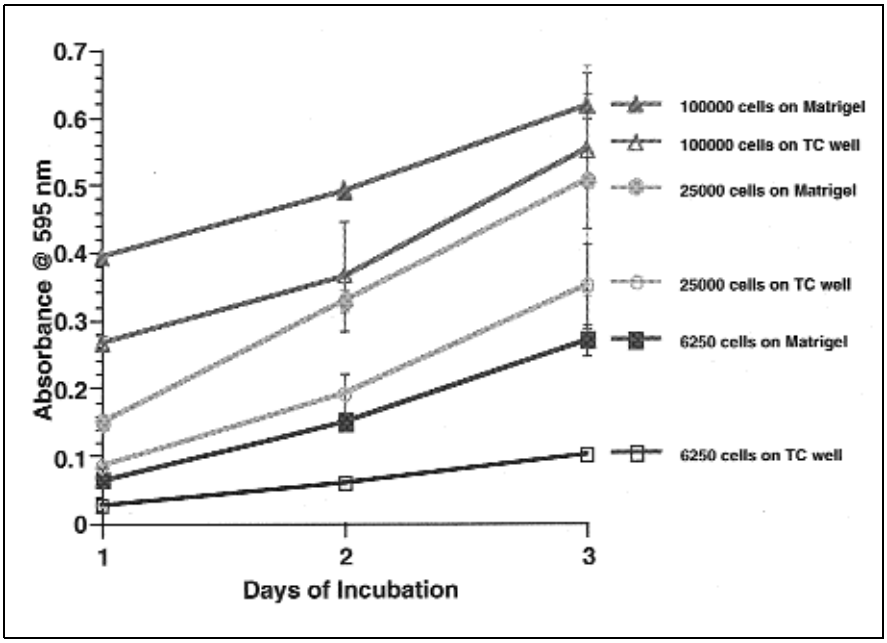

Figure 1. Growth and quantitation of viable MDA.MB.231 cells on Matrigel. (A) Growth curves of MDA.MB.231 cells grown on either tissue culture (TC) wells (uncoated) or on wells coated with Matrigel (MG). Data points and error bars represent the average and standard deviations of triplicate samples.

CA, USA). Background absorbance values from wells containing only Matrigel (typically ≤0.02 U) were subtracted from sample absorbance values containing cells. Values and error bars represent the average and standard deviation, respectively, of triplicate samples. Each experiment was repeated at least three times with essentially similar results.

Matrigel Tumor Outgrowth Assay

The ability of tumor cells to spread and invade basement membrane was determined by a modification of methods described previously (4). Briefly, 0.3 mL of Matrigel (10 mg/mL) was added to 24-well microplates and incubated 15 min at 37°C for gelation. Cells were harvested with 0.125% trypsin containing 2 mM EDTA, counted and added to each well (6.25×10^3 to 1×10^5, as indicated in the figure legends). After overnight incubation in 5% CO_2 at 37°C, the tetrazolium dye MTT (0.5 mg/mL) was added. Microscopic analysis of the cellular network morphology was determined using an inverted microscope for 2 h after incubation with MTT in 5% CO_2 at 37°C. The image was captured with an Image-1™ Visual System (Universal Imaging, West Chester, PA, USA) and digitally photographed with a liquid crystal display (LCD) camera.

Cellular Migration and Invasion

To quantitate cell invasion, the modified Boyden chamber method was used (1). Briefly, 2×10^5 cells were added to 8-µm filters (Nucleopore®; Corning Costar, Cambridge, MA, USA) pre-coated with collagen I (5 µg/filter) or collagen I (5 µg/filter) overlayed with Matrigel (10 µg/filter) and incubated for 5 h at 37°C in modified Boyden chambers using fetal bovine serum (10%) in DMEM in the lower chamber as a chemo-attractant. Filters were removed, fixed and stained with Hema 3® solutions (Curtin Matheson Scientific, Houston, TX, USA) and those cells that had migrated through the filters were quantitated by light microscopy at high power (400×). The number of cells per high-powered field (5 fields per filter) was determined from duplicate filters. The experiments were repeated three times with essentially similar results.

RESULTS AND DISCUSSION

MTT Cell Growth and Viability Assay on Matrigel

The principle behind the MTT assay is the conversion of MTT to formazan dye crystals by mitochondrial hydrolase present in viable cells. The crystals are solubilized with detergent, and the absorbance of the dye is measured by spectrophotometry. Because Matrigel consists of tumor extracts of basement membrane and contains various growth factors (9), it is possible that the Matrigel could affect the conversion of MTT by mitochondrial hydrolase or affect the sensitivity of the spectrophotometric measurements. Therefore, we compared the ability of the MTT assay

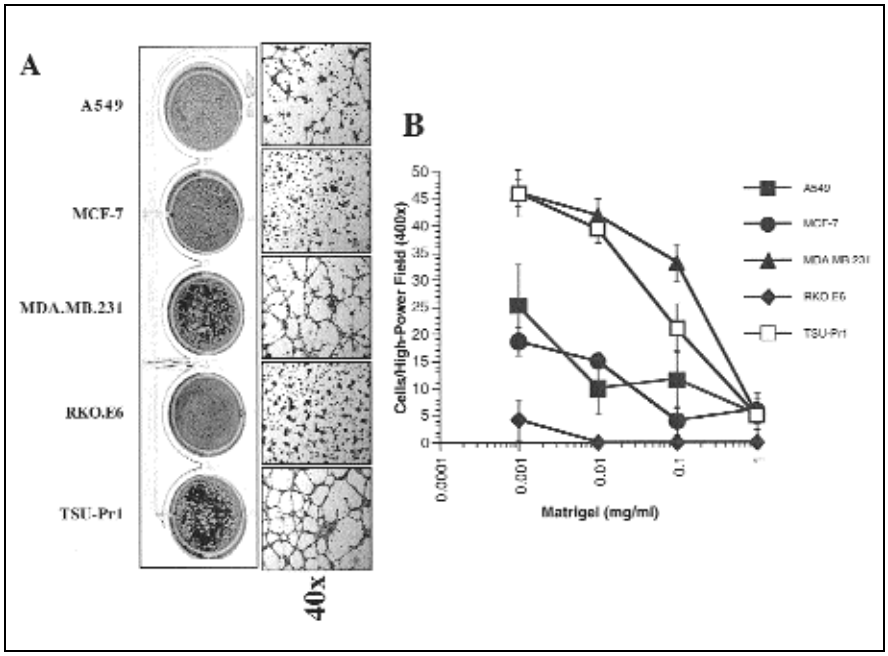

Figure 2. Cellular outgrowth with the Matrigel-MTT assay compared to invasion with the modified Boyden chamber. (A) Several cell lines (100 000/well) were cultured on Matrigel for 24 h and then stained with MTT for 2 h. Visual inspection of the cells (left panels) is compared with 40× magnification images (right panels). (B) Quantitation of cell invasion (200 000 cells/chamber) after 5 h with the modified Boyden chamber. In general, cells that aggregated on Matrigel were noninvasive (RKO) or poorly invasive (MCF-7). Cells that formed extensive outgrowth on Matrigel (MDA.MB.231 and Tsu-pr1) were also highly invasive. The A549 cells formed limited outgrowths on Matrigel and were moderately invasive.

Table 1. Cytotoxicity Effects of Various Drugs on MDA.MB.231 Cells Cultured on Matrigel

Drug	Stock Concentration	Drug Dilution (Percent Survival) 10^{-5}	10^{-4}	10^{-3}
Doxorubicin	10 mg/mL	69.1 ± 0.6	45.4 ± 8.0	51.6 ± 6.1
Etoposide	20 mg/mL	92.5 ± 5.8	105.7 ± 10.7	74.6 ± 1.4
Suramin	100 mg/mL	104.1 ± 2.3	93.4 ± 2.2	111 ± 11.7
Taxol	23 mM	83 ± 3.1	83 ± 1.8	87.6 ± 1.4
Minocycline	10 mg/mL	95.9 ± 8.7	114.9 ± 18.7	106.7 ± 4.2

Percent survival was determined from triplicate samples after 24 h using drug dilutions (10^{-5}, 10^{-4}, 10^{-3}) prepared from the indicated stock concentrations.

to distinguish the growth characteristics of the mammary tumor cell line MDA. MB.231 on tissue culture plastic and on dishes coated with Matrigel. As shown in Figure 1, MDA.MB.231 cells grown on either tissue culture wells or wells coated with Matrigel proliferated when incubated for 72 h. The rate of proliferation was dependent on the number of cells and whether the cells were grown on Matrigel or on uncoated wells. In general, the proliferation rate was greater when the cells were grown on Matrigel. Thus, the MTT assay can be used to assess cell viability on Matrigel.

Comparison of the MTT/Matrigel Outgrowth Assay with the Modified Boyden Chamber Using Invasive and Noninvasive Cells

Previous methods of visualizing cells on Matrigel have involved microscopy. The lack of contrast between unstained cells and Matrigel often makes documentation of invading cells and cellular network formation difficult. Furthermore, one could not determine cell viability. Cells incubated with MTT on Matrigel showed intracellular localization of the dye without

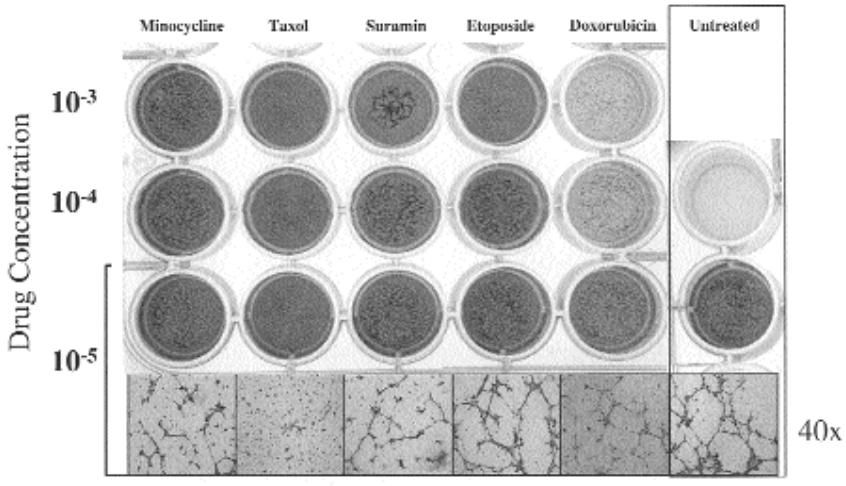

Figure 3. Effect of drug treatment on cellular outgrowth. MDA.MB.231 cells (100 000/well) were treated with taxol, suramin, etoposide, minocycline or doxorubicin for 24 h at the indicated drug dilutions from stock (Table 1). Networks were visualized macroscopically after MTT staining (top panels). 40× magnification verifies network formation (bottom panels).

increased background staining (Figure 2A). Network formation occurred within 6 h in the absence of cell division. Increased cell numbers after 24–48 h resulted in thickening of the networks (data not shown). Networks were evident with 1×10^5 cells after 24 h and with 2.5×10^4 cells after 72 h. Previous reports demonstrating the culture of endothelial or mammary epithelial cells on Matrigel in vitro and the formation of cellular networks and polarized organotypic spheres resembling acini (1–3,9) have facilitated investigations in cellular physiology. In combination with the MTT assay to enumerate viable cells and visualize cell morphology, this method allows the macroscopic visualization of cellular network formation on Matrigel.

Several noninvasive and invasive cancer cell lines were analyzed to determine if the assay could distinguish among these cells. By adding the MTT dye into the Matrigel invasion assay, visual inspection alone without magnification could determine that MDA.MB.231, A549 and Tsu-pr1 cells were highly invasive, as indicated by the extensive cellular network formation (Figure 2A, left panels). In contrast, MCF-7 and RKO.E6 cells aggregated and did not spread on Matrigel. Confirmation was obtained at 40× magnification. The levels of MTT converted by the cells were comparable with absorbance $(A)_{595}/A_{595}$ values of 0.4–0.45 for all cell lines, indicating that the numbers of viable cells were similar. Therefore, combining the Matrigel invasion and MTT reduction assay allows a quick method to compare the invasive properties of the cells on Matrigel.

To verify and quantitate the invasive capacity of these cell lines using another well-established invasion assay, the modified Boyden chamber assay was used (Figure 2B). MDA.MB.231 and Tsu-pr1 cell lines were highly invasive, whereas the RKO.E6 cell line was not. A549 and MCF-7 were moderately invasive. This is in agreement with other reports, which have shown that the ability of cellular network formation correlates with invasiveness (1). Thus, under these conditions and with these cell lines, the Matrigel-MTT assay is capable of distinguishing noninvasive from invasive cell lines as well as the modified Boyden chamber assay. Using modified Boyden chambers or in vivo animal tests to screen cell lines would be costly and time-consuming, whereas the MTT-Matrigel method is rapid and inexpensive. The extensive use of animals in initial screenings would also be avoided.

Screening of Anti-Invasive Chemotherapeutic Agents

Inhibition of metastasis using agents that prevent invasion or migration of tumor cells from their original site is a common approach in cancer therapeutics (8). To test whether the Matrigel-MTT assay could be used to screen various drugs for their anti-metastatic properties, MDA.MB.231 cells were seeded into wells containing Matrigel with or without various chemotherapeutic agents. Cellular networks were visually apparent in untreated cells (Figure 3). Taxol and doxorubicin effectively inhibited network formation. Closer examination of the wells with 40× magnification confirmed the macroscopic observations that only taxol and doxorubicin prevented cellular network formation. Because the toxicity of these drugs could artificially disrupt network formation, the absorbances of the formazan product in the wells were determined. As indicated in Table 1, none of the drugs except for doxorubicin was significantly toxic. Therefore, the inhibition of cellular network formation by taxol was not due to toxicity. Doxorubicin was toxic but did not affect cellular network formation at lower doses (Figure 3). Doxorubicin has been described as a genotoxic agent, whereas taxol has been shown to abrogate microtubule formation, which affects cellular proliferation and motility. Like doxorubicin, etoposide, a topoisomerase inhibitor, was cytotoxic but did not inhibit network formation at low doses. Networks were disrupted with high doses of all the drugs except minocycline. Interestingly, the highest dose of suramin treatment resulted in cellular aggregation (Figure 3), but not cytotoxicity (Table 1) consistent with the known anti-growth factor properties of this drug. It is possible that the chemotherapeutic action of suramin involves cell aggregation, thereby inhibit-

ing metastasis. Similar findings were obtained with the use of another invasive carcinoma cell line, Tsu-pr1 (data not shown). Taken together, the ability to distinguish between drugs with cytotoxic and anti-invasive or anti-migratory properties can be achieved with the use of the Matrigel-MTT assay. The cost of screening various agents with this method is minimal in comparison with other methods. Alternative methods are far better-suited than this method for quantitating inhibition of invasion. Nonetheless, this method allows the screening of numerous pharmacological or natural compounds for anti-invasive properties.

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