0023-6837/02/8201-109$03.00/0
LABORATORY INVESTIGATION

Vol. 82, No. 1, p. 109, 2002
Printed in U.S.A.

BRIEF METHOD

A Novel Fluorometric Invasion Assay for Quantitative Determination of Tumor Cell Invasion

Hiroyoshi Yoh, Sonshin Takao, and Takashi Aikou

The First Department of Surgery, Kagoshima University School of Medicine, Kagoshima, Japan

Tumor cell invasion into the basement membrane is an important step in the multistep process of cancer metastasis (Liotta, 1986). Various in vitro assay systems have been developed for the investigation of tumor cell invasion into the basement membrane. These methods primarily employ a reconstituted basement membrane matrix (Matrigel) as an invasive substrate coated on a microporous filter membrane within a Boyden chamber (Albini et al, 1987; Schlechte et al, 1990). In such systems, establishing a uniform horizontal layer of Matrigel is difficult. In addition, after careful removal and fixation of the filter, counting the number of invaded cells under a high-powered microscope is time consuming, laborious, and sometimes subjective. To overcome such disadvantages, we devised an invasion 3-(4, 5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with a uniform Matrigel layer prepared by a water-repellent treatment with paraffin and a tetrazolium-based colorimetric MTT assay for counting the invaded cells (Imamura et al, 1994). However, with this procedure, accurate quantitation of cell invasion requires the careful removal of noninvaded cells from the surface of the Matrigel layer using a cotton swab.

We then developed a fluorometric invasion assay for quantitative determination of tumor cell invasion that uses a fluorescence-blocking membrane insert (Falcon HTS FluoroBlok insert, Becton Dickinson Labware, Franklin Lakes, New Jersey), as well as a uniform Matrigel layer produced by a paraffin-treated water-repellent method. The fluorescence-blocking microporous membrane was developed by Tchao (1997).

In this assay cells are fluorescently labeled with a 3', 6'-(Di (*O*-acetyl)-2', 7'-bis[*N*, *N*-bis (carboxymethyl) aminomethyl]) fluorescein, tetraacetoxymethyl ester (calcein-AM) (Dojindo Lab., Kumamoto, Japan) in the invasion assay chamber at the end of the experimental period, thus avoiding potential cytotoxicity problems over the long incubation period of the assay. The number of invaded cells can be quantified, with no requirement for removing the noninvaded cells, through the use of a fluorescence-blocking membrane, which blocks almost all of the fluorescence apart from a very low level of background through the micropores.

For water-repellent treatment, a block of paraffin was rubbed on the inside wall of the insert and residue was removed with a cotton swab. The water-repellent–treated FluoroBlok inserts were sterilized with ethylene oxide gas and placed into 24-well tissue culture plates (FIA chamber). For Matrigel coating, 70 μl of 0.2 mg/ml Matrigel was applied to the membrane filters of the insert. Filters were dried overnight (for more than 12 hours) in a laminar flow hood.

The outline of our assay is shown in Figure 1. Conditioned medium (700 μl), obtained by incubating NIH3T3 cells for 24 hours in serum-free medium (Ajinomoto Co., Tokyo, Japan) was then placed in the lower well as a chemoattractant. Culture media (200 μl) was gently added to the upper well to reconstitute the Matrigel layer and to provide growth medium for the cells. Finally the FIA chamber with medium (inner) and attractant (outer) was incubated for 60 minutes before cell addition. To the inner well, 100 μl of cell suspension resuspended in culture medium at a density of 1×10^6 cells/ml (1×10^5 cells) was added. After incubation for 72 hours, the FIA chamber was gently rinsed with PBS to wash out the remaining serum. Subsequently 700-μl and 300-μl aliquots of calcein-AM solution at a concentration of 2 μg/ml in PBS were added into the outer and inner wells, respectively. After a 60-minute incubation, the calcein-AM solution was exchanged with PBS to reduce the background fluorescence. The fluorescence in the FIA chamber was measured immediately from the bottom of the plates using a Cytofluor4000TC fluorescent multiwell plate reader (PerSeptive Biosystems, Framingham, Massachusetts) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm (gain is 50).

Received October 18, 2001.
This study was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture, and the Ministry of Health and Welfare, Japan.
Address reprint requests to: Dr. Hiroyoshi Yoh, The First Department of Surgery, Kagoshima University School of Medicine, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. E-mail: yohdr@po.synapse.ne.jp

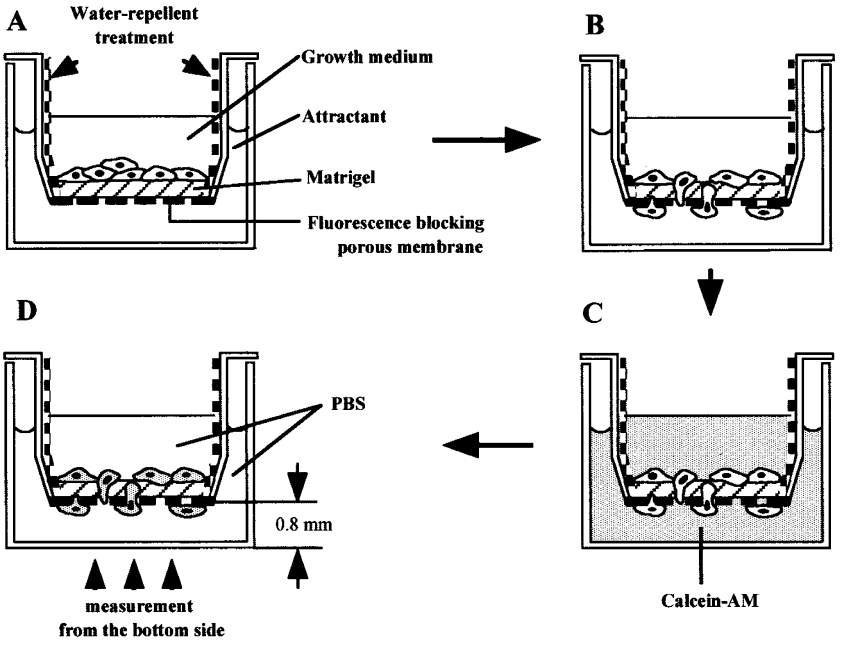

Figure 1.
A, Tumor cells (1×10^5 cells) are seeded into the upper well of the FIA chamber in 100 μl culture medium. The chamber is incubated at 37° C in an atmosphere of 5% CO_2. B, After 72 hours of incubation, invasive cells have passed through the Matrigel layer onto the surface of the fluorescence-blocking membrane. C, Fluorescent labeling by calcein-AM after rinse. D, After 60 minutes of incubation, the fluorescence of the invaded cells under the fluorescence-blocking membrane is measured from the bottom side of the plate. The number of invaded cells is calculated from the fluorescence of the same cells measured before the assay.

Fluorescence intensity depends quantitatively on the optical pathlength and solute concentration, as well as on the excitation source intensity and fluorescence collection efficiency of the instrument used. The excitation pathlength from the bottom side of the plate to the surface of the insert of the FIA chamber is 0.8 mm. The fluorescence measured on a 96-well microplate was proportionally higher than that measured for an insert in a 24-well plate due to differences in the optical pathlengths, and the ratio of the fluorescence was 1.669. Accordingly, the number of invaded cells and the percent-

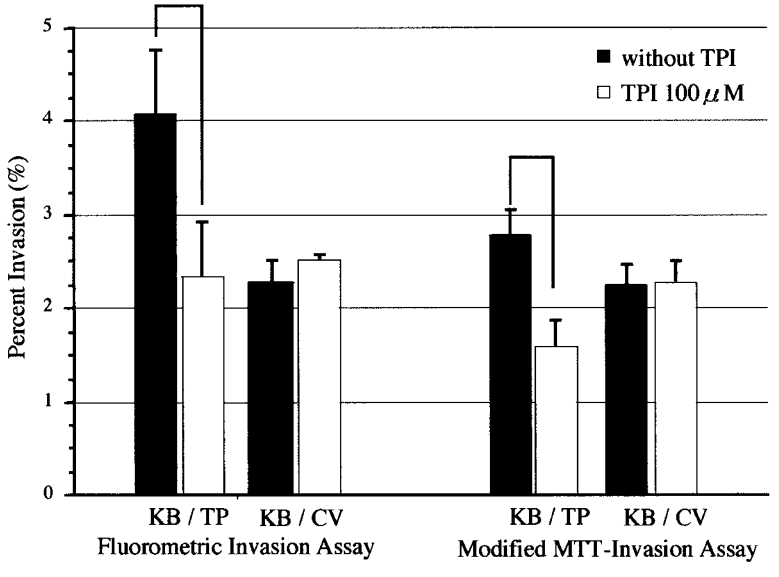

Figure 2.
The percentage of invasion value calculated for the eight cell lines was significantly higher in the fluorometric invasion assay than in the invasion 3-(4, 5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay procedure. The sensitivity of the fluorometric method was statistically higher than that of the MTT method ($p = 0.016$, $p = 0.042$, respectively). Statistical significance was determined using the Mann-Whitney U test (mean $\pm$ SD, $n = 3$). Both assays indicate that thymidine phosphorylase inhibitor (TPI) prevents the invasion of the KB/TP cells, but not that of the KB/CV cells. This result indicates that the fluorometric invasion assay can be used for screening potential anti-invasive drugs.

age of invasion (PI) were calculated using the fluorescence value obtained from 1×10^4 cells measured on a 96-well microplate before FIA using the following formula:

PI (%) = the number of invaded cells/the number of seeded cells $\times$ 100 = [measured fluorescence on the FIA chamber]/[fluorescence of 1×10^4 cells] $\times$ 16.69.

The FIA is also superior to the MTT invasion assay with respect to reproducibility, convenience of assay, and speed of performance (60 minutes for the FIA compared with 4 hours for the MTT assay) between the PI values of KB/TP and KB/CV cells, a cell line model system that shows the impact of PD-ECGF/thymidine phosphorylase on tumor invasiveness.

In addition, we examined the applicability of the novel FIA assay procedure for drug screening compared with the invasion MTT assay. For this study, we examined the effect of thymidine phosphorylase inhibitor (TPI), a known anti-invasive agent, on tumor cell invasion (Furukawa et al, 1992; Takao et al, 2000). Both assay methods indicate that TPI prevents the invasion of KB/TP cells, but not the invasion of KB/CV cells. However the FIA screening of TPI as an anti-invasive agent was both more sensitive and more convenient than the MTT assay (Fig. 2). These data indicate that the FIA provides fast and reproducible quantification of invaded cells and will be useful in the screening of potential anti-invasive drugs.

In conclusion, the fluorometric invasion assay provides a simple, accurate, and reproducible in vitro assay for the quantification of tumor cell invasion. This assay is applicable not only for determining the invasiveness of tumor cells but also for screening potential anti-invasive agents.

References

Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, and McEwan RN (1987). A rapid *in vitro* assay for quantitating the invasion potential of tumor cells. Cancer Res 47:3239–3245.

Furukawa T, Yoshimura A, Sumizawa T, Hraguchi M, Akiyama S, Fukui K, Ishizawa M, and Yamada Y (1992). Angiogenic factor. Nature 356:668.

Imamura H, Takao S, and Aikou T (1994). A modified invasion-3-(4, 5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumor cell invasion. Cancer Res 54:3620–3624.

Liotta LA (1986). Tumor invasion and metastasis-role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res 46:1–7.

Schlechte W, Brattain M, and Boyd D (1990). Invasion of extracellular matrix by cultured colon cancer cells: Dependence on urokinase receptor display. Cancer Commun 2:173–179.

Takao S, Akiyama S, Nakajou T, Yoh H, Kitazono M, Natsugoe S, Miyadera K, Yamada Y, and Aikou T (2000). Suppression of metastasis by thymidine phosphorylase inhibitor. Cancer Res 60:5345–5348.

Tchao R (1997). U.S. Patent Number 5,601,997, effective February 11, 1997.