A Modified Invasion-3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium Bromide Assay for Quantitating Tumor Cell Invasion

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ABSTRACT
Reconstituted basement membrane matrix (Matrigel) has been utilized for in vitro assay of tumor cell invasion in recent years. In the conventional chamber for the invasion assay, however, a large number of cells passed easily through the center of the Matrigel-coated filter because the Matrigel layer could not be completely uniform by the meniscus formation. To prevent the meniscus phenomenon of the Matrigel layer, we devised a water-repellent treatment of the inside wall of the assay chamber with paraffin. Consequently, very few erythrocytes passed through the Matrigel-coated filter of this modified chamber with the erythrocyte assay, which was used to demonstrate the evenness and uniformity of the Matrigel layer on the filter. For quantitating a small number of cells which invaded through the Matrigel-coated filter by the invasion assay, a tetrazolium-based colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay was used. The invasive abilities of the eight different cell lines were determined by this invasion assay, and the Matrigel-coated filter by the invasion assay, a tetrazolium-based colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay was used. The invasive abilities of the eight different cell lines were significantly exceeded the normal cell lines in the percentage of invasion (P < 0.01). Therefore, the modified invasion assay using a large size Transwell chamber with the use of a small amount of Matrigel. We have often observed that cells passed easily through the Matrigel-coated filter without invasion because of the uneven thickness or absence of Matrigel. Therefore, we devised a simple, easily reproducible in vitro assay for quantitating tumor cell invasion.

INTRODUCTION
Tumor metastasis is a multistage process (1) in which the basement membrane plays a critical role as a barrier against invasion (2, 3). In the study of tumor invasion and metastasis, it is thus important to investigate the relationship between tumor cells and the basement membrane (4–7). Four kinds of connective tissues, the bladder wall (8, 9), amnion (10–12), lens capsule (13), and chicken chorioallantonic membrane (14), have been used to assess the invasion of tumor cells in vitro. Such systems are difficult to standardize and somewhat complicated. To make the more quantitative and reproducible invasion assay, the reconstituted basement membrane has been developed in assay systems under various experimental conditions (15–21). In 1986, Terranova et al. (15) used a pressed disc composed of laminin and type I and type IV collagens resembling the basement membrane to assay the invasion of tumor cells in a Boyden chamber. Kleinman et al. (16) reported the development of a reconstituted basement membrane, Matrigel (17). The following year, Albini et al. (18) devised an in vitro procedure, assay tumor invasion using Matrigel in a Boyden chamber. In 1989, Repesh et al. (20) presented a new assay system, which used radio-labeling for quantitating tumor cell invasion, as a simpler way for evaluation of tumor cell invasion using a Transwell chamber with Matrigel. In 1983, on the other hand, Mossmann reported a quantitative colorimetric method, MTT3 assay (22). In 1990, Schlechte et al. (23) applied the MTT assay to the invasion assay using a large size Transwell chamber. The uniformity of the coated Matrigel, however, was not examined well. It is difficult to coat an even and uniform Matrigel layer on the filter of the assay chamber with the use of a small amount of Matrigel. We have often observed that cells passed easily through the Matrigel-coated filter without invasion because of the uneven thickness or absence of Matrigel. Therefore, we devised a simple, easily reproducible in vitro assay for quantitating tumor cell invasion.

MATERIALS AND METHODS
Agents. Matrigel (Collaborative Research Co., Bedford, MA), a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, was prepared. Its major components are laminin, collagen type IV, and heparan sulfate proteoglycan. In this study, Matrigel concentrations of 1.0 and 0.2 mg/ml, diluted 10- and 50-fold with cold distilled water, were used. A conditioned medium, obtained by incubating NIH3T3 cells for 24 h in serum-free medium (Azinomoto Co., Tokyo, Japan), was used as a chemo-attractant. MTT (Sigma Chemical Co., St. Louis, MO) was used as a colorimetric assay (22, 24, 25) and dimethyl sulfoxide (Nacalai Tesque, Inc., Kyoto, Japan) was used to dissolve formation crystals of MTT.

Cells. Seven cell lines, NFSaY83, BALB/3T3 clone A31, NIH3T3/14-1, MRC-5, MRC-5 SV1 TG1, Vero, and HeLa, and a primary culture of normal human umbilical endothelial cells were used. The NFSaY83 cell line, provided by Dr. K. Ando, was derived from a fibrosarcoma that had highly metastatic potential to the lung (26). The BALB/3T3 clone A31 cell line derived from a BALB/c mouse embryo contained fibroblast-like cells with contact inhibition. The NIH3T3/14-1 cell line was a spontaneous transformant of NIH3T3 without contact inhibition. Both cell lines were provided by Riken Cell Bank (Tokyo, Japan) and were maintained in minimum essential medium Eagle’s. The MRC-5 cell line, composed of fibroblast-like cells, was derived from normal lung tissue (27); and the MRC-5 SV1 TG1 cell line, composed of a SV40 transformant of MRC-5, was provided by Riken Cell Bank. These cells were maintained in a mixed medium of equal parts of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture. Vero cells derived from the renal tissue of a normal African monkey (28) were a normal epithelium-like cell line with contact inhibition and were also provided by Riken Cell Bank. HeLa and Vero cells were maintained in minimum essential medium Eagle’s. All of the culture media were supplemented with 10% heat-inactivated fetal bovine serum with penicillin (100 units/ml) and streptomycin (100 μg/ml).

Water-repellent Treatment of Assay Chamber. A Chemotaxicell chamber (Kubota Co., Tokyo, Japan) with a filter 8 mm in diameter, similar to a Transwell chamber (Costar Co., Cambridge, MA) with a filter 6.5 mm in diameter, was used for this study (Fig. 1A). The filter was a polyvinylpyrrolidone-free polycarbonate membrane with 8-μm pores (Nucleopore). The water-repellent treatment of a Chemotaxicell chamber with paraffin was performed to prevent the meniscus phenomenon (Fig. 1B). A block of paraffin was rubbed on the inside wall of this chamber, and its residue was removed using a cotton swab. This chamber was used as the modified assay chamber in this study.

Matrigel Coating. The 1.0-mg/ml Matrigel was applied on the membrane filters of the Chemotaxicell chambers with the paraffin treatment and was also applied on the membrane filters of the Chemotaxicell chambers without the paraffin treatment. This was repeated using the 0.2-mg/ml Matrigel. The filters

3 The abbreviation used is: MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide.

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3620
RESULTS

Uniformity of the Matrigel Layer. In a Chemotaxicell chamber without the water-repellent treatment as the conventional assay chamber, the Matrigel layer showed a meniscus-like form during the coating process, thick at the periphery and thin at the center (Fig. 3, left). Following the water-repellent treatment, however, Matrigel formed an even and uniform layer on the filter (Fig. 3, right). In the erythrocyte assay using the conventional assay chamber, a large number of erythrocytes passed through the 1.0-mg/ml Matrigel filters and the 0.2-mg/ml Matrigel filters. With the 0.2-mg/ml Matrigel, which created an adequate condition for tumor invasion, the mean percentage of the erythrocytes that fell into the lower well was over 5% in all of the different amounts of diluted Matrigel. In the modified assay chamber, however, no erythrocytes passed through the Matrigel-coated filter and fell into the lower well with the use of any volume of the 1.0-mg/ml Matrigel or 70 to 100 μl of the 0.2-mg/ml Matrigel. Erythrocytes were separated from peripheral blood obtained from healthy volunteers. A total of 1 × 10^7 erythrocytes in 200 μl of RPMI 1640 were seeded onto the Matrigel-coated filters of the conventional and modified assay chambers. After 24 h of incubation, the number of erythrocytes that passed through the filter and fell into the lower well were counted under the microscope.

Invasion-MTT Assay. This assay was carried out using the methods described by Repesh (20) and Schlechte et al. (23) but with modifications. The modified Chemotaxicell chambers with water-repellent treatment were used as the upper wells, and each filter was coated with 70 μl of a 50-fold dilution (0.2 mg/ml) of Matrigel which was a required condition for tumor cell invasion. After the invasion assay, the MTT assay was used for quantitating the invaded cells through the Matrigel-coated filter (Fig. 2).

Percentage of Invasion. The percentage of invasion was calculated to compare the invasive potential of the 8 different cells after the modified invasion-MTT assay using the following formula. A standard curve was calculated from the absorbance of 1 × 10^6 cells.

\[
\text{Percentage of Invasion} = \frac{\text{Absorbance of invaded cells}}{\text{Absorbance of seeded cells}} \times 100
\]

Statistical Analysis. Student's t test was used for statistical analysis.
ASSAY FOR QUANTITATING TUMOR CELL INVASION

MRC-5 and human umbilical endothelial cells, was very low and showed no increase in 72 h. The difference of absorbance between malignant and normal cell lines showed the maximum levels after

Fig. 5. Macroscopic views of the erythrocytes (A, B, E, F) or NIH3T3/14-1 cells (C, D, G, H) assay on the uniformity of the Matrigel-coated filter after 24 h (A, B, C, D) and 72 h (E, F, G, H) of incubation. The conventional (A, C, E, G) and modified (B, D, F, H) chambers were used. The filter of each chamber was coated with 70 μL of the 0.2-mg/ml Matrigel. The part of the Matrigel-coated filter where the erythrocytes or NIH3T3/14-1 cells passed through appeared as a white area or as dark spots by MTF reaction, respectively. In the conventional chambers, both the erythrocytes and NIH3T3/14-1 cells invaded through it appearing as the scattered pattern of dark spots after 72 h (H).

Fig. 6. Relationship between the number of BALB/3T3 clone A31 cells and absorbance by MTT assay. This cell line was used as a normal cell line; also very few cells passed through the Matrigel-coated filter of the modified chamber. The absorbance was directly proportional to the number of cells within the range of 600 to 2 × 10⁴ cells/well using an enzyme-linked immunosorbent assay reader. Each point is the mean ± SD (bars) of five experiments. Error bars not shown were contained within the symbols.
CONVENTIONAL CHAMBER WITHOUT THE WATER-REPELLENT TREATMENT, A LARGE DISCUSSION

The Matrigel layer was not coated on the filter uniformly, the eryth

mity of a Matrigel layer on the filter. Erythrocytes derived from

by protein staining (18—20) and microscopic observation (18, 19). In

assay was 72 h. The adequate incubation time for this

absorbance. The difference between the absorbance of malignant and normal cell lines

was the most significant after 72 h of incubation. Each point is the mean ± SD (bars) of triplicate determinations. Error bars not shown were contained within the symbols.

percentage of invasion of the malignant cell lines exceeded those of the normal cell lines, and it was significantly higher than that of normal cell lines (C) (P < 0.01). Columns, means of triplicate determinations; bars, SD.

72 h of incubation (Fig. 8). The adequate incubation time for this assay was 72 h.

Percentage of Invasion. The percentage of invasion of the malignant cell lines exceeded those of the normal cell lines, and it was significant statistically (P < 0.01) (Fig. 9).

DISCUSSION

The uniformity of the Matrigel layer on the filter has been certified by protein staining (18—20) and microscopic observation (18, 19). In this study, the erythrocyte assay was useful for evaluating the uniformity of a Matrigel layer on the filter. Erythrocytes derived from human peripheral blood are about 7.5 μm in diameter. Therefore, if the Matrigel layer was not coated on the filter uniformly, the erythrocytes could pass easily through some parts of the filter. In the conventional chamber without the water-repellent treatment, a large amount of erythrocytes passed through the center of the filter and fell into the lower well. This result indicates that the Matrigel layer on the filter of the conventional chamber was not uniform and even due to the meniscus phenomenon. It has been reported that 1% or less of tumor cells have invasive ability in a tumor mass (19, 20). If over 0.1% of erythrocytes passed through the Matrigel-coated filter, this invasion assay would be unsuitable for determining the number of invaded cells. As the result of the erythrocyte assay using the modified chamber, however, the number of erythrocytes that passed through the Matrigel-coated filter decreased markedly to less than 0.02%. The uniformity of Matrigel coating in the modified chamber was also confirmed by the differences of the invasive pattern between the conventional and the modified chamber using NIH3T3/14-l and Vero cells. These results indicate that the water-repellent treatment of the inside wall of the assay chamber with paraffin was effective for preventing the meniscus phenomenon and for making the uniform Matrigel coating.

In the conventional invasion assay using Matrigel, the invaded cells were quantified by a visual counting under the light microscope (15, 18, 19) and by assessing the number of radiolabeled cells (20). In 1989, Hendrix et al. (29) reported that the visual assessment was as good as the radiolabeled method and was easier to use. However, it has been difficult to accurately evaluate the invasive abilities of malignant cells. A MTT assay described by Mossman (22) in 1983 is a quantitative colorimetric method that uses the activity of the mitochondrial dehydrogenase and the absorbance of the dark blue product formazan. Therefore, this assay is directly proportional to the number of living cells. Schlechte et al. (23) has applied a MTT assay to the invasion assay using the conventional large chambers. In this study, a MTT assay was used to obtain the accurate evaluation for measuring the invaded cells. A problem in applying the MTT assay was that the high absorbance of the background would interfere with the estimation of a small number of invaded cells, but the problem was solved by dissolving the formazan with dimethyl sulfoxide using another 24-well culture plate for a MTT reaction. Thus, the modified chamber and MTT assay were useful for quantitating the invaded cells accurately and quickly.

The invasiveness depended on the concentration and volume of Matrigel. Using either the 1.0-mg/ml Matrigel or at least 80 μl of the 0.2-mg/ml Matrigel, the tumor cells could not invade through the Matrigel-coated filter. In this study, the adequate concentration and volume of Matrigel were 0.2 mg/ml and 70 μl, respectively. Furthermore, the incubation time also was an important factor for the inva-
assay. The malignant cells revealed almost no invasion into the Matrigel-coated filter for the first 5 to 24 h of incubation, similar to the observations of Kramer et al. (21). After 48 h of incubation, the absorbance of malignant cells increased gradually with time; then after 72 h of incubation, the difference in absorbance between the normal and malignant cells was obvious. These results indicate that the tumor cell invasion into the Matrigel layer depends on the incubation time.

The percentage of invasion was useful for comparing the invasive abilities of various kinds of cell lines in this study. The percentage of invasion of malignant cell lines was significantly higher than that of normal cell lines. Noel et al. (30), however, have reported that the invasion into Matrigel was not correlated to the malignant metastatic cell phenotype by microscopic study. Further study concerning the relationship between the metastatic potential and the percentage of invasion is now in progress using the modified invasion-MTT assay.

REFERENCES

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