A Modified and Convenient Method for Assessing Tumor Cell Invasion and Migration and Its Application to Screening for Inhibitors

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In order to screen potent inhibitors of tumor invasion and metastasis, we here devised a simple and reproducible *in vitro* assay for tumor invasion and migration. A conventional cell-counting assay using a Transwell chamber with a microporous membrane filter is troublesome and time-consuming, involving visually counting the cells under a microscope, and the invaded or migrated cells are sometimes distributed unevenly in predetermined fields on the lower surface of the filter. Therefore, it is difficult to evaluate the invasive and migratory abilities of tumor cells easily and quantitatively by the cell counting method. In the present study, crystal violet dye was used for staining the invaded cells and colorimetrically assessing the invasive ability per filter as an absorbance. In this crystal violet assay, tumor cell invasion into a reconstituted basement membrane Matrigel was proportional to both the cell number added into the chamber and the incubation period, and inversely proportional to the amount of Matrigel barrier on the upper surface of filter. The results obtained by this dye-uptake method were highly consistent with those of a conventional cell-counting assay. Using this crystal violet assay, the anti-invasive effect of doxorubicin (DOX) was detected more easily and found to be highly proportional to that by the conventional cell-counting method. We therefore applied this convenient assay method to screen anti-invasive and anti-metastatic compounds. As a result, caffeic acid was found to be more active in the inhibition of both tumor cell invasion and migration without showing direct cytotoxicity *in vitro* than other related compounds.

Key words metastasis; Transwell; dye-uptake method; caffeic acid; invasion assay

Tumor invasion through the extracellular matrix (ECM) and basement membrane (BM) is a crucial step in the complex, multi-stage process of tumor metastasis. This process consists mainly of tumor cell adhesion, migration and enzymatic degradation of the ECM and BM.^{1–4)} Therefore, many attempts have been made to find potent inhibitors of tumor cell invasion and migration from various candidates as one of the strategies for eradicating cancer metastasis.

Recently, either a Transwell cell culture chamber or a Boyden chamber with a microporous membrane, which is precoated with some ECM components and a reconstituted BM (Matrigel), has been utilized to assess tumor cell invasion and motility under various experimental conditions *in vitro*.^{5,6)} To obtain more quantitative and reproducible results of tumor invasion, some improvements in the assay system have also been made such as radiolabeling⁷⁾ or colorimetric methods,⁸⁾ in replacement of a microscopic counting method, or a water-repellent treatment for a uniform coating of Matrigel.⁹⁾ In the present study, we established a simple, more easy and non-hazardous *in vitro* assay system for tumor cell invasion and migration and applied this method to screen anti-invasive compounds.

MATERIALS AND METHODS

Cells A highly metastatic human HT-1080 fibrosarcoma and murine B16-BL6 melanoma cells were maintained in Eagle's minimum essential medium (EMEM: Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 5% heat-inactivated fetal bovine serum (FBS: Gibco Laboratories, Life Technologies, Inc., Grand Island, NY), 2 mM glutamine, 50 IU/ml

penicillin and 50 µg/ml streptomycin.

Agents Caffeic acid (3,4-dihydroxycinnamic acid), coumaric acid (4-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), isoferulic acid (3-hydroxy-4-methoxycinnamic acid) and cinnamic acid were purchased from Tokyo Kasei Organic Chemicals, Tokyo, Japan. Eugenol (4-allyl-2-methoxyphenol) was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. These compounds were dissolved in dimethylsulfoxide at the concentration of 20 mM. Doxorubicin (DOX) was purchased from Kyowa Hakko Co., Ltd., Tokyo, Japan and served as a positive control.

Assay for the Invasion and Migration of Tumor Cells The invasive activity of HT-1080 or B16-BL6 cells was assayed in Transwell cell culture chambers with a membrane filter (Costar, Cambridge, MA) as described previously,¹⁰⁾ with some modifications. Briefly, the inside wall of the chamber was treated with paraffin to obtain a water-repellent property for an even and uniform coating of Matrigel on the polyvinylpyrrolidone-free polycarbonate filter.⁹⁾ The filters, with 8.0-µm pore size (Nucleopore, Pleasanton, CA), were precoated with 1 µg of fibronectin (FN: Iwaki Glass, Tokyo, Japan) on their lower surfaces and dried at room temperature. A reconstituted BM Matrigel (containing laminin, type IV collagen, heparan sulfate proteoglycan and entactin: Collaborative Research Co., Bedford, MA) was applied to the upper surfaces of the filters (5 µg/10 µl/filter), and dried in the same way. These prepared filters were washed extensively in phosphate buffered saline (PBS) and dried just before use. Exponentially growing tumor cells were harvested by 1 mM EDTA in PBS, washed three times with FBS-free EMEM and resuspended to the density of 2.5×10^5 cells/ml in the

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culture medium with or without compounds. The cell suspension was added into the upper compartment of the chamber in triplicate and incubated for appropriate hours at 37 °C in an atmosphere of 5% CO₂ and 95% air.

In the cell migration assay, the lower surfaces of the filters were precoated only with FN without coating of the upper surfaces with Matrigel. The subsequent procedures were carried out similarly to those in the invasion assay.

Cell Counting Assay The tumor cells on the filters were fixed with methanol and then stained with hematoxylin and eosin as described previously.¹¹⁾ The remaining cells on the upper surface of the filter were removed by wiping it with a cotton swab. The cells that invaded to the lower surface through the Matrigel and a filter were counted under a microscope in five predetermined fields at a magnification of 400.

Crystal Violet Staining Assay After fixation with methanol, the filters were stained with 0.5% crystal violet in 20% methanol for 30 min. After gentle rinsing with water, the cells on the upper surfaces of the filters were removed in the same way as described above. The filters containing the stained cells that had invaded to their lower surfaces were removed from the Transwell chambers and individually transferred to a separate well in a 96-well culture plate. The crystal violet dye retained on the filters was extracted with 30% acetic acid and the absorbance was colorimetrically measured at 590 nm.

RESULTS

A Convenient Invasion Assay Using Crystal Violet Staining It is troublesome and time-consuming to visually count the number of invaded cells in five predetermined fields per filter under a microscope using the conventional invasion assay. Therefore, a simple and easy assay has been sought to assess tumor invasion quantitatively and rapidly for screening effective substances. We here applied a well-known crystal violet staining method for assessing cell numbers¹²⁾ to this assay and evaluated the number of cells which invaded to the lower surfaces of filters.

We first investigated the relationship between the number of cells added into the chamber and the absorbance of crystal violet in the invaded cells on the lower surface of a filter pre-coated with FN and Matrigel. Figure 1 shows that the absorbance was increased according to the cell number added into a chamber (ranging 0.25–25 × 10⁴ cells/chamber). This result indicates that the number of invaded cells per filter was fairly associated with the number of cells added into a chamber. Considering the guaranteed linearity of optical density in this colorimetric measurement, 2.5 × 10⁴ cells/chamber was employed as the added cell density in the following experiments.

Tumor cell invasion through the reconstituted BM may also be determined by the thickness (amount) of the Matrigel layer and the incubation time. As shown in Fig. 2, the absorbance in the lysate of the invaded HT-1080 cells after a 4-h incubation was inversely related to the amount of Matrigel coated on the upper surface of the filter (0–50 µg/filter). Similar results were also observed using metastatic B16-BL6 melanoma cells.

Fig. 1. Relationship between the Absorbance of Crystal Violet in the Invaded Cells and the Number of Tumor Cells Added into a Chamber

The invasive activity of HT-1080 cells was assayed in Transwell chambers with microporous membrane filters precoated with Matrigel (5 µg) and FN (1 µg) on their upper and lower surfaces, respectively. Various numbers of tumor cells were added into the upper compartments of the chambers and incubated for 4 h at 37 °C. The filters were fixed and stained with 0.5% crystal violet in 20% methanol. After gentle rinsing, the remaining cells on the upper surface of the filter were removed by wiping with a cotton swab. The stained cells on the filter were extracted and colorimetrically assessed by measuring its absorbance at 590 nm. The data were expressed as the mean ± S.D. of triplicate cultures.

Fig. 2. Effect of the Thickness of the Matrigel Layer on the Invasion of Tumor Cells

The filters of Transwell chambers were pre-coated with Matrigel (5–50 µg/filter) and FN (1 µg) on their upper and lower surfaces, respectively. HT-1080 cells (2.5 × 10⁴ cells/0.1 ml, ■) or B16-BL6 cells (2 × 10⁵ cells/0.1 ml, □) were added into the upper compartment of the chamber and incubated for 4 h at 37 °C. The numbers of invaded cells were assessed and shown in Fig. 1. The data were expressed as the mean ± S.D. of triplicate cultures.

When HT-1080 cells were incubated in the chamber for various time periods, the number of invaded cells onto the lower surface in a cell-counting assay and the amount (absorbance) of crystal violet in the invaded cell lysate in this dye uptake assay method were observed to increase time-dependently and similarly to each other (Fig. 3). According to these results, this crystal violet assay method is suggested to be useful for determining the number of invaded cells per filter.

Reliability of the Crystal Violet Assay Method In order to confirm the reliability of this assay, we examined the effect of an anti-cancer agent, DOX, as a positive control on the invasion of HT-1080 cells. Figure 4 shows that DOX reduced the number of invaded cells and the ab-

Fig. 3. Time Dependency of the Invasion of HT-1080 Cells through Matrigel

The filters of transwell chambers were pre-coated with Matrigel (5 μ g/filter) and FN (1 μ g) on their upper and lower surfaces, respectively. HT-1080 cells (2.5×10^4 cells/0.1 ml) were added into the upper compartment of the chamber and incubated for various hours at 37 °C. The numbers of invaded cells were assessed by either the crystal violet method (●), or the conventional cell-counting method (○). The data were expressed as the mean $\pm$ S.D. of triplicate cultures.

Fig. 4. Inhibition of the Invasion of HT-1080 Cells by DOX

The invasive activity of HT-1080 cells was assayed in Transwell chambers, as shown in Fig. 3. HT-1080 cells (2.5×10^4 cells/0.1 ml) were added into the upper compartment of the chamber and incubated with or without DOX for 4 h at 37 °C. The numbers of invaded cells were assessed by either conventional cell-counting (○) or the crystal violet method (●). The data were expressed as the mean $\pm$ S.D. of triplicate cultures.

sorbance of crystal violet in the invaded cell lysate in both the cell counting and the crystal violet assays, respectively. The effects obtained by both assays were quite similar and dependent on the concentration of DOX. Several independent experiments showed that this anti-invasive effect of DOX was reproducible (data not shown).

Inhibition of the Invasion and Migration of HT-1080 Cells by Caffeic Acid and Its Related Compounds
In order to search for candidate substances with potent anti-invasive activity, we first attempted to examine the effect of six chemically defined compounds derived from natural medicinal plants (shown in Fig. 5) on HT-1080 cell invasion and migration by using the crystal violet assay. As shown in Fig. 6, caffeic acid (2 mM) as well as DOX (0.086 mM) were found to remarkably inhibit the invasion of HT-1080 cells (the inhibition rates: 92% and 55%, respectively). However, other compounds at the concentra-

Fig. 5. Chemical Structures of Caffeic Acid and Its Related Compounds

Fig. 6. Effect of Caffeic Acid and Its Related Compounds on the Invasion of HT-1080 Cells in the Crystal Violet Assay

The invasive activity of HT-1080 cells was assayed in Transwell chambers, as shown in Fig. 3. HT-1080 cells (2.5×10^4 cells/0.1 ml) were added into the upper compartment of the chamber and incubated with or without caffeic acid, its related compounds or DOX for 4 h at 37 °C. The numbers of invaded cells were assessed by the crystal violet assay method, as shown in Fig. 1. The data were expressed as the mean $\pm$ S.D. of triplicate cultures.

tions used did not affect the tumor invasion. Similar effects on the migration of HT-1080 cells to the FN-coated filter (without a coating of Matrigel) were observed as shown in Fig. 7. These compounds, differently from DOX, rarely affected the growth of HT-1080 cells or the cell viability at the concentrations used in this study (data not shown). These results suggest that the anti-invasive effect by caffeic acid may be partly associated with the inhibition of tumor cell migration. Since caffeic acid was much more effective at inhibiting tumor cell invasion and migration than other related compounds, a relationship between their structures

Fig. 7. Effect of Caffeic Acid and Its Related Compounds on the Migration of HT-1080 Cells in the Crystal Violet Assay

The migratory activity of HT-1080 cells was assayed in Transwell chambers with microporous membrane filters pre-coated with FN (1 μ g) on their lower surfaces. HT-1080 cells (2.5×10^4 cells/0.1 ml) were added into the upper compartment of the chamber and incubated with or without caffeic acid, its related compounds or DOX for 4 h at 37 °C. The numbers of migrated cells were assessed as shown in Fig. 1. The data were expressed as the mean $\pm$ S.D. of triplicate cultures.

and the anti-invasive activity might be postulated to exist.

DISCUSSION

Radiolabeling⁷⁾ and MTT assays⁸⁾ have been used to improve the troublesome and time-consuming problems of the conventional cell-counting invasion assay and to evaluate the number of invaded cells. However, these advanced assay systems are still hazardous to humans and the environment, and several improvements have been required. In the present study, crystal violet dye¹²⁾ was used for staining the invaded cells on the lower surfaces of filters and colorimetrically assessing the invasive ability. We here devised a simpler and easier *in vitro* assay for assessing tumor cell invasion and migration, then applied this assay to screen effective inhibitors of tumor invasion and metastasis. The absorbance of crystal violet in the invaded cell lysate increased with increases in the number of tumor cells added into a chamber and with the incubation period (Figs. 1 and 3), while it was inversely proportional to the thickness of the Matrigel barrier on the upper surfaces of filters (Fig. 2). Reduction of the absorbance of crystal violet in the invaded cell lysate by DOX was highly consistent with the decrease in the number of invaded cells assessed by a cell-counting method (Fig. 4). All the results indicate that this newly established crystal violet assay may be simple, reliable and useful both for assessing the tumor cell invasion and migration and for screening anti-invasive substances.

Using this convenient assay, caffeic acid was found to be more active in terms of the inhibition of both tumor cell invasion and migration than other related compounds. Considering the chemical structures of caffeic acid and its related compounds (shown in Fig. 5), an undefined moiety(s) in caffeic acid may be important for the expression of its anti-invasive property. In addition, since

caffeic acid did not affect the tumor cell growth and viability *in vitro* at the concentration used in this study, the anti-invasive effect of caffeic acid may be associated with the inhibition of tumor cell migration, which is considered to be a crucial step in the invasive process. Caffeic acid has been reported to inhibit the secretion of tumor necrosis factor- α and interleukin-1 β from lipopolysaccharide-stimulated whole blood cells,¹³⁾ tumor promotion induced by a phorbol ester,¹⁴⁾ xanthine oxidase activity¹⁵⁾ and colon carcinogenesis.¹⁶⁾ In addition, caffeic acid is able to induce apoptosis and alter the redox state in transformed rat fibroblasts.¹⁷⁾ Thus, the anti-invasive and anti-migratory effects of caffeic acid might be related to such biological properties. We are now planning to examine the inhibitory mechanisms of tumor cell invasion and migration in detail.

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