Development of a Novel Cell-based Assay to Evaluate the Malignant Potential of Cancer *In Vitro*

CHIASA HIRAKAWA[1,2*], KUNIHIKO TANAKA[1*], YOSHIHIRO TAKAYA[1], SHINSUKE NAKAGAWA[1,4], YASUFUMI KATAOKA[2,4], YUTAKA TAGAWA[3,4] and MASAMI NIWA[1,4]

[1]*Department of Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523;*
[2]*Department of Pharmaceutical Care and Health Sciences, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nakakuma, Jonan-ku, Fukuoka, 814-0180;*
[3]*Department of Nursing, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8520;*
[4]*PharmaCo-Cell Co. Ltd., 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

Abstract. *Background: Metastasis is a leading cause of cancer death. To evaluate the complex metastatic process in vitro, an attempt was made to develop a cell-based assay (Can kit) that could evaluate the late stages of metastasis. Materials and Methods: Two membrane chambers were set up of which the upper membrane chamber with 8 μm pores was covered with normal cell layers. Cancer cells were introduced to the upper chamber and after passing through the normal cell layers dropped through onto the lower chamber membrane where cancer colonies formed and were evaluated based on the reduction of transepithelial electrical resistance (TEER) with a Madin-Darby canine kidney (MDCK) cell monolayer. Results: When two pairs of cancer cell lines, with different metastatic potentials in vivo, were applied to the Can kit assay, differences in potentials between the two cell lines in vitro were demonstrated. The reduction in the TEER was correlated with the total area of the cancer colonies and the production of matrix metalloproteinases (MMPs). Conclusion: A cell-based assay able to evaluate the malignant potential of cancer in vitro was developed and is considered to be useful for research and the clinical examination of cancer metastatic potential.*

*Both authors contributed equally to this study.

Correspondence to: Kunihiko Tanaka, MD, Ph.D., Department of Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel: +81 958197043, Fax: +81 958197044, e-mail: kunny-ta@net.nagasaki-u.ac.jp

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Metastases, rather than primary tumors, are responsible for approximately 90% of all cancer deaths (1, 2). The metastatic process consists of a series of complex stages (3-5). When the diameter of a primary tumor exceeds 1-2 mm, it needs to develop a blood supply that can support its growth and metabolism (6). This process is called angiogenesis. The new blood vessels also provide an escape route through which cancer cells detach from the primary tumor and enter the circulation, a process known as intravasation. Ultimately, most circulating cancer cells die owing to anoikis. However, surviving cancer cells can arrest in the capillary vessels of distant organs, adhere to the endothelial cells and extravasate into the surrounding tissue. If the cancer cells adapt to the microenvironment in the secondary sites, they grow and form a metastatic nodule.

The comprehensive evaluation of the metastatic potential of cancer is difficult because of the complex metastatic process. A number of researchers have studied this process using inoculation of cancer cells into immunodeficient mice to investigate the metastatic process because this represents the whole process of metastasis. However, attempts to study this process *in vivo* have presented some difficulties in terms of orthotopic grafts in mice and the continuous observation of cancer cells. Furthermore, long periods of time are needed to obtain a result. On the other hand, a few *in vitro* assays have been used to evaluate the malignant potential of cancer. Each *in vitro* assay can generally only evaluate one stage of metastasis. For example, the Matrigel invasion assay can evaluate only the potential for invasion, even though the assay involves adhesion to the extracellular matrix, proteolysis and chemotactic migration (7). Therefore, the development of an *in vitro* assay that can accurately determine the malignant potential of cancer, particularly the

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metastatic potential, is required to advance cancer research and help to reduce the number of cancer deaths.

In this study, a new cell-based assay (Can kit) was developed to reconstruct the latter stages of metastasis *in vitro*, which include the adhesion of cancer cells to endothelial cells, extravasation, and growth of the metastatic nodule. The mechanism of the reduction of transepithelial electrical resistance (TEER) was also investigated using the colony formation of cancer cells and the expression of matrix metalloproteinase (MMP) -2 and -9 in the Can kit.

Materials and Methods

Cell culture. NIH3T3 (murine embryonic fibroblast cell line) was purchased from RIKEN BioResorce Center (Tsukuba, Ibaragi, Japan). GP8.3 (rat brain endothelial cell line) was a gift from Dr. Maria A. Deli. MDCK NBL-2 (Madin-Darby canine kidney cell line), B16-F0 and B16-F10 (murine malignant melanoma cell lines) and SW480 and SW620 (human colon cancer cell lines) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA), 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA) and 10 μg/ml Mycokill AB (PAA Laboratories GmbH, Pasching, Austria). The cells were used for experiments within 10 passages. All the cell lines were grown in a humidified incubator with 95% air, 5% CO_2 at 37˚C. The two pairs of cancer cell lines have known different malignant potentials *in vivo*. The B16-F0 cells have low metastatic potential and the B16-F10 cells have high metastatic potential, and the SW480 cells have no metastatic potential and the SW620 cells have high metastatic potential *in vivo*.

Can kit protocol. The Can kit comprised two membrane chambers, a 12-well plate and NIH3T3, GP8.3 and MDCK cells. The upper chamber contained a Chemotaxicell filter with 8 μm pores (Kurabo, Osaka, Japan) and the lower chamber was a 12-well Transwell with 0.4 μm pores (#3460; Corning Inc., Lowell, MA, USA) through which cells could not pass. On day 1, 2.4-2.5×10^5 NIH3T3 fibroblast cells were plated and cultured on the reverse side of the membrane of the upper chamber. After 6 hours, the upper chamber was set in a 24-well plate and cultured for 2 days. The NIH3T3 cells were used as the feeder layer for the GP8.3 endothelial cells and the cancer cells, and as the physiological barrier against the migration of the GP8.3 cells. A total of 1.7-3.0×10^5 GP8.3 cells were seeded on the observation side of the membrane in the upper chamber and cultured. Thus, the upper chamber was equivalent to a blood vessel and the environment below the upper chamber was equivalent to a metastatic site.

After cultivation for 1 day, 2.5-3.0×10^5 cancer cells were seeded in the upper chamber and it was placed on the lower chamber in a 12-well plate. The next day, the upper chamber was removed and the cells that had dropped through into the lower chamber were further cultured for 2 days. Then 2.0×10^5 MDCK cells were seeded in the lower chamber, and the TEER was measured after 7-16 days (Figure 1).

TEER measurement. TEER was measured with a planar electrode chamber, which was connected to an EVOM Epithelial Voltohmmeter (World Precision Instruments, Berlin, Germany). The background resistance (Transwell without cells) was subtracted from the resistance

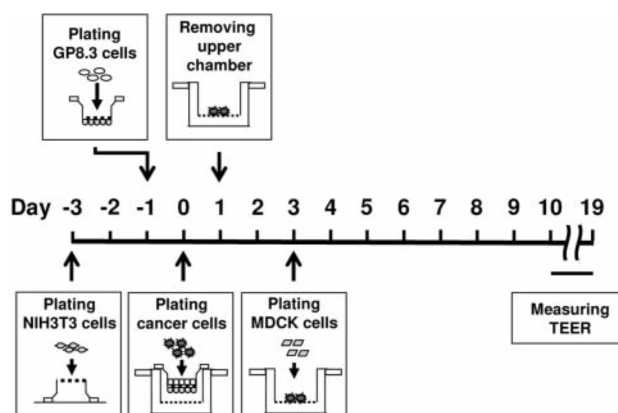

Figure 1. *Protocol of the cell-based assay (Can kit). NIH3T3 cells are cultured on the reverse side of the upper chamber membrane with 8 μm pores. After 2 days, GP8.3 cells are cultured on the membrane. The next day, cancer cells are plated in the upper chamber and the double chamber was placed in a 12-well plate. Some cancer cells penetrate the upper chamber membrane, covered with the normal cell layers, and drop onto the lower chamber membrane. The next day, the upper chamber is removed and MDCK cells are plated on the lower chamber membrane 2 days later. After the MDCK cells form a complete monolayer with the cancer colonies, the TEER (transepithelial electrical resistance) is measured.*

of the samples. TEER of the MDCK monolayer exceeding 6,000 $\Omega \cdot cm^2$ was considered to represent an intact epithelium. Samples without seeding of cancer cells in the upper chamber were used as controls. At least two independent experiments were conducted for each cell line.

Colony evaluation. Cancer cell colony formation on the lower chamber membrane was assessed after the TEER was measured. The total area of cancer colonies per well was estimated under an inverted microscope at ×100.

Gelatin zymography. Gelatin zymography was performed as described in the manufacturer's protocol (Millipore, Billerica, MA, USA). In brief, the conditioned medium in the lower chamber of the Can kit was changed to 100 μl of serum-free medium after the TEER measurement and colony evaluation. After culture for 1 day, 15 μl of the conditioned medium was mixed with 5 μl of sample buffer (0.5 M Tris-HCl, pH 6.8, 40% glycerol, 0.01% bromophenol blue and 8% SDS) and then incubated for 10 min at room temperature. The samples were electrophoresed on a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Nacalai Tesque, Kyoto, Japan). After electrophoresis, the gel was incubated in 2.5% Triton® X-100 for 30 min, washed in the developing buffer (0.05 M Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$) for 30 min and incubated at 37˚C overnight. The gel was stained with 0.5% Coomassie brilliant blue R250 for 30 min and destained with 50% methanol and 10% acetic acid in water. The clear bands resulting from digestion of the substrate by gelatinase were then visualized.

Statistical analysis. All the experiments were performed at least in triplicate. Student's *t*-test was used to compare the means of the groups. A *p*-value of <0.05 was considered to indicate a statistically significant difference.

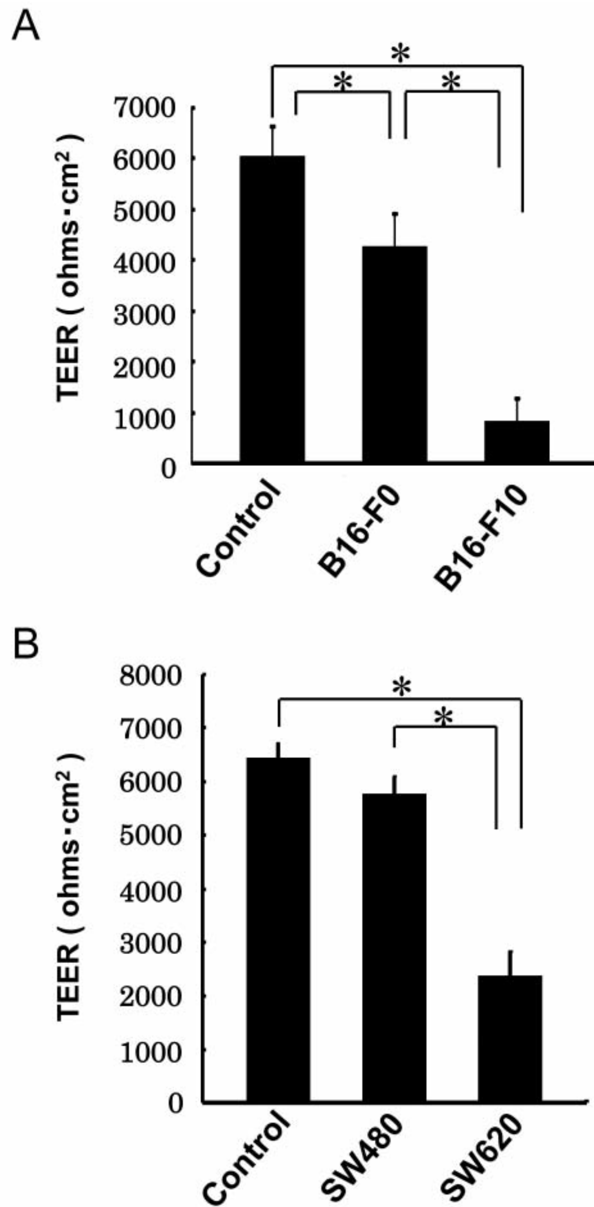

Figure 2. *TEER measurements of two pairs of cancer cell lines (A) B16-F0 and B16-F10 and (B) SW480 and SW620. TEER (ohms·cm²; ohms × surface area) was calculated by subtracting the resistance of a cell-free lower chamber membrane and correcting for surface area. Values are means±SE (n=3). *p<0.05.*

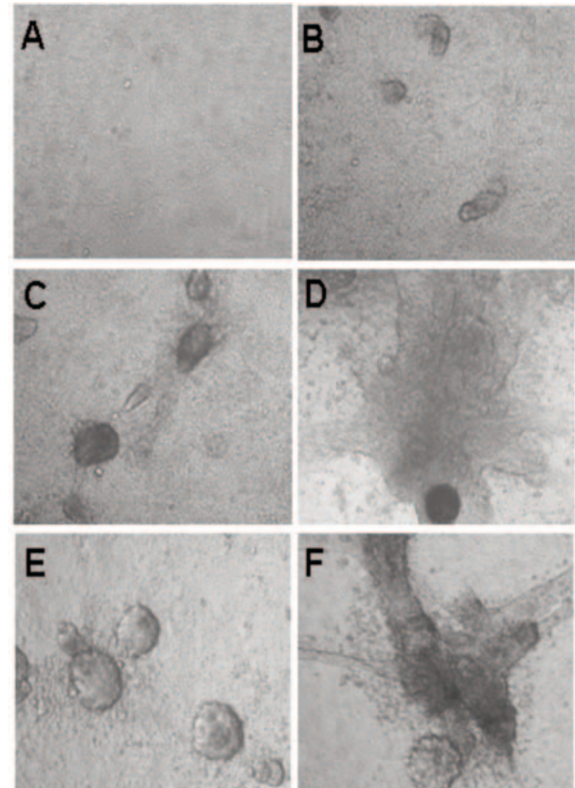

Figure 3. *Cancer colonies on the lower chamber membrane of the Can kit observed under an inverted microscope. (A) MDCK cells plated on the new lower chamber membrane without the other cells; (B) Control in which cancer cells were not seeded on the upper chamber of the Can kit; (C) B16-F0; (D) B16-F10; (E) SW480 and (F) SW620 cells. Original magnification ×100.*

Results

TEER measurements. As shown in Figure 2A, both the B16-F0 and B16-F10 cells significantly reduced the TEER compared with the controls (control: 6040±590 Ω·cm2; B16-F0: 4273±625 Ω·cm²; B16-F10: 829±432 Ω·cm²). The B16-F10 cells showed a more significant reduction in the TEER than did the B16-F0 cells.

The SW620 cells significantly reduced the TEER, whereas the SW480 cells did not cause a significant reduction in TEER compared with the control cells (control: 6433±159 Ω·cm²; SW480: 5753±202 Ω·cm²; SW620: 2350±278 Ω·cm²; Figure 2B). There was also a significant difference in the TEER between the SW480 and the SW620 cells.

Colony evaluations. To clarify the mechanism by which the TEER is reduced by the presence of the cancer cells, the cancer cell colony formation and area on the membrane of the lower chamber were evaluated. Compared with the MDCK monolayer (Figure 3A), even the control samples showed round colonies (Figure 3B) and they were considered to be GP8.3 cells or NIH3T3 cells because cancer cells were not seeded in the control tests. The B16-F0 cells formed round colonies with lateral branches (Figure 3C), whereas the B16-F10 cells formed ameba-like colonies with lateral branches (Figure 3D). The total area occupied by the colonies of B16-F10 was four times larger than that of B16-F0. The

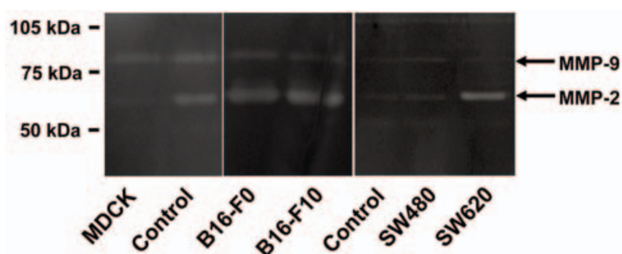

Figure 4. *Gelatin zymography of the conditioned media from the Can kit. Conditioned media in the lower chamber was electrophoresed on 10% SDS-PAGE containing 1 mg/ml gelatin. Clear bands resulting from digestion by gelatinase were visualized by Coomassie staining. The left and middle pictures are from the same gel at the same time.*

SW480 cells formed round colonies (Figure 3E) while the SW620 cells formed oval colonies with long lateral branches (Figure 3F). The total area occupied by the colonies of SW620 was three times larger than that of SW480.

Gelatin zymography. MMP-2 and MMP-9 were detected in the conditioned medium from the control sample. MMP-9 activity was detected in the conditioned medium from the MDCK cells, but the MMP-2 activity was much lower (Figure 4, left). The activity of MMP-9 in the conditioned media from the B16-F0 and B16-F10 samples was less than that of the control sample. The activity of MMP-2 in the conditioned medium from the B16-F10 sample was greater compared with that in the control and B16-F0 samples (Figure 4, middle). Although the MMP-9 activity in the conditioned medium from the SW480 sample was slightly greater than those of the control and the SW620 samples, MMP-2 activity in the conditioned medium from the SW620 sample was greater compared with that in the control and SW480 samples (Figure 4, right).

Discussion

In the present study, some of the disseminated cancer cells with high malignant potential adhered to the endothelial cells, invaded the layer of endothelial cells and fibroblast cells passing through the 8 μm pores, and then dropped into the lower chamber. A few endothelial cells and fibroblast cells also dropped into the lower chamber. We have previously reported that some of the cancer cells that drop from the membrane can survive (8). It has also been reported that the dropped cancer cells are more malignant than the parent cells (9, 10). Observations of the cancer colonies suggested that the colonizing abilities in the Can kit were correlated with the malignant potential of the cancer. When the TEER was measured at the end of the culture period, many cancer cells were dead and floating in the lower chamber (data not shown). Penetration through the normal cell layers and the membrane pores, and dropping into the medium was most probably a difficult process for the cancer cells, and only those with high malignant potential and resistance to anoikis could form substantial colonies on the lower chamber membrane.

The TEER of the total cells on the lower chamber membrane was measured when the MDCK cells had formed a complete monolayer. MDCK cells have been reported to have a high TEER when they form a monolayer because their tight junctions are very rigid (11). The results of an electrical resistance breakdown assay in which cancer cells induced breakdown of the TEER when they were co-cultured with a MDCK monolayer were previously reported (12, 13). This principle was applied in the Can kit.

The murine malignant melanoma cell line B16-F10 was established by repeated injection of tumor cells into nude mice and recovering the cells from pulmonary tumor nodules *in vivo* (14). It has been reported that B16-F10 is more metastatic compared with the parental cell line B16-F0 in pulmonary colonization (15). When these two cell lines were tested with the Can kit, both cell lines showed malignant potential, but the B16-F10 cells were more malignant than the B16-F0 cells based on the magnitude of the TEER reduction. The present data were thus consistent with the data of the metastatic potential *in vivo* of these cell lines. Interestingly, Gehlsen *et al.* described that, of the B16 cell lines selected for variant metastatic behavior *in vivo*, B16-F1 and B16-F10 had similar invasion profiles on an invasion assay *in vitro* (16). Therefore the present assay was able to evaluate the strength of the malignant potential of these cancer cells, which the invasion assays could not determine.

The human colon cancer cell lines SW480 and SW620 were respectively derived from a primary tumor and lymph node metastasis resected from a single patient (17). It has been reported that when these two cell lines were inoculated into nude mice, the SW480 cells did not form a secondary tumor in the liver (no metastatic potential), but the SW620 cells did (high metastatic potential) (18, 19). When SW480 and SW620 cells were evaluated using the Can kit, the SW620 cells significantly reduced the TEER, but the SW480 cells did not. These data were therefore also consistent with the *in vivo* data. Although many researchers have reported that SW620 cells are more invasive than SW480 based on the results of invasion assays (20-22), Kusakai *et al.* showed the opposite results using the Matrigel invasion assay (23). The studies indicate that the invasion assays are influenced by the experimental conditions and that appropriate experimental conditions were used in the Can kit.

MMPs are known to play a crucial role in cancer spread and invasion due to the degradation of the extracellular matrix and basal membrane. Gelatin zymography showed that MMPs, particularly MMP-2, were involved in the reduction of the TEER in the Can kit. When the SW480 and

SW620 cells were evaluated without NIH3T3, the MMP-2 activities of both cell lines were barely detectable (data not shown). Che *et al*. reported that the MMP-2 activity of oral squamous carcinoma cells was increased when they were co-cultured with fibroblast cells (24). The data suggested that the fibroblast cells played an important role not only as the feeder layer, but also as a microenvironment for the cancer cells in the Can kit.

In conclusion, a novel cell-based assay has been developed that can evaluate the late stages of metastasis *in vitro*. In this assay, the reduction of the TEER was correlated with the area occupied by the cancer colonies and with MMP production in the lower chamber. The present assay is easy to handle and requires less time compared with *in vivo* experiments. Therefore, we believe the Can kit will be useful for a range of applications both in research and clinical settings.

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