*Original Article*

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# Invasion of carcinoma cells into reconstituted type I collagen gels: Visual real-time analysis by time-lapse microscopy

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**Summary** **Stromal-epithelial interactions play a critical role in promoting tumorigenesis and invasion. To obtain detailed information on cancer cell behaviors on the stroma and kinetics of cell migration, which cannot be observed by conventionally-used Boyden chamber assays, this study was aimed at analyzing the cell invasion process *in vitro* using time-lapse microscopic observation. Serum-free conditions and reconstituted type I collagen gels which provided a basal membrane-stroma-like microenvironment were used to first establish a basal condition. Time-lapse microscopic observation for 30 h of cell invasion into the collagen gel revealed kinetic parameters and individualistic behavior of cancer cells. Of breast cancer MDA-MB-231 or MCF-7 cells and colon cancer LS180 or HT29 cells examined, MDA-MB-231 cells most rapidly disappeared from the collagen gel surface under basal conditions. Estrogen-dependent MCF-7 cells disappeared at a rate approximately two times slower than that of MDA-MB-231 cells under serum- and phenol red-free conditions. By the addition of 10 nM β-estradiol to the basal medium, MCF-7 cell invasion was facilitated to a rate similar to that of MDA-MB-231 cells. Microscopic analyses of collagen gel-sections demonstrated that most of the MDA-MB-231 and MCF-7 cells remained within 60 μm from the gel top under basal conditions, which is consistent with the observation obtained using Boyden chambers that no cells could cross the collagen I gel barrier unless 1% fetal calf serum was added to basal conditions. In summary, this study demonstrated future applicability of this method to understand the initial phase of cancer cell invasion processes.**

***Keywords:*** Cancer cell invasion, reconstituted type I collagen gel, time-lapse microscopy, real-time analysis

## 1. Introduction

Biological behavior of epithelial cells is restricted by the surrounding microenvironment such as the basement membrane (BM) and cell-cell interaction (*1-3*). The basement membrane is a thin extracellular matrix (ECM) that underlies epithelia and separates cells from the stroma. Malignant cells must cross this membrane to invade stroma and eventually establish distant metastases. Complex interactions between cancer cells and regulatory factors within the tumor microenvironment function cooperatively to control tumor cell invasion and eventual metastasis. Several genetic and epigenetic factors, both in the cell and in the microenvironment, contribute to the progression of cancer cells towards metastases. The escape of cancer cells from primary to distant sites is first established by producing proteases (*4*) that degrade ECM, consisting of basement membrane and stromal components. In addition, hormones and growth factors may also regulate motility and invasion of some types of carcinoma such as breast carcinoma. It has been shown that acquisition of cell invasion ability requires epithelial-to-mesenchymal transition (EMT) and that

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induction of EMT in breast carcinoma cells can be associated with hormones such as estrogen (*5*).

Of *in vitro* models currently available, those using Matrigel as a biologically active basement membrane model for *in vitro* invasion assays have been used for two decades (*6,7*). In those assays, tumor cells are placed in the upper chamber of a Boyden migration chamber. The upper and lower chambers are separated by a porous membrane coated with Matrigel. A chemoattractant in the lower well stimulates migration. After an interval, tumor cells are recovered from or counted on the lower surface of the membrane. Although useful and convenient, the Boyden chamber assay cannot provide detailed information on cell invasion parameters such as kinetics of cell migration and migration behavior of cells on the BM-stroma in the presence of stimuli. In order to study cell invasion processes under different conditions reflecting the *in vivo* situation, we developed a real-time assay system that should allow direct visualization and assessment of cancer cell invasion processes.

Reconstituted gels with type I collagen, a major component forming a lattice structure in stroma (*8*), were used in the present study to observe migration of colorectal and breast carcinoma cells. That is, cancer cells were inoculated on a reconstituted 0.08% collagen gel (1.2 mm-thick) in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 0.1% Bovine Serum Albumin (BSA) (the basal medium) and how the cells disappeared from the visible field were observed under a time-lapse phase contrast microscope. As the first step, the basal assay system in the absence of fetal calf serum (FCS) and phenol red, which mimics estrogen effects (*9*), was used to obtain information on the basal invasion of the tested cancer cells. Once the basal condition was established, effects of various parameters on invasion, such as changes in ECM components and/or addition of stimulants, can readily be assessed.

Time-lapse microscopic observation for 30 h of cell invasion into the collagen gel revealed kinetic parameters and individualistic behaviors of various cancer cells. Of breast cancer MDA-MB-231 or MCF-7 cells and colon cancer LS180 or HT29 cells examined, MDA-MB-231 cells most rapidly disappeared from the collagen gel surface under the basal conditions. Estrogen-dependent MCF-7 cells disappeared at a rate approximately two times slower than that of MDA-MB-231 cells under serum- and phenol red-free conditions. The effect of estrogen on invasion of estrogen-dependent MCF-7 breast carcinoma cells was then evaluated, which revealed that MCF-7 cell invasion was facilitated by the addition of 10 nM β-estradiol to the basal medium and had a rate similar to that of MDA-MB-231 cells. These results demonstrated future applicability of this real-time analysis to understand the cancer cell invasion processes.

## 2. Materials and Methods

### 2.1. *Cell lines and cell culture conditions*

Human breast carcinoma cells (MDA-MB-231 and MCF-7) and human colon carcinoma cells (LS180 and HT-29) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 and MCF-7 cells were maintained in low glucose DMEM containing 5% FCS (Sigma-Aldrich Japan, Tokyo, Japan) and high glucose DMEM containing 10% FCS, respectively, supplemented with penicillin, streptomycin, and 2 mM glutamine. LS180 cells were cultured in Minimum Essential Medium (MEM) containing 10% FCS. HT-29 cells were cultured in McCoy's 5A Medium (Invitrogen, Carlsbad, CA, USA) containing 10% FCS. These cells were cultured at 37°C in a 5% $CO_2$ atmosphere.

### 2.2. *Preparation of a collagen gel bed for observation of cancer cell invasion*

Type I collagen (collagen I) extracted from bovine skin by acetic acid was purchased from Koken Co. Ltd., Tokyo, Japan. To construct a collagen gel bed, 0.1% collagen I in 1 mM HCl was mixed with 10 times-concentrated phenol red-free DMEM, and neutralized with 100 mM HEPES (collagen I : DMEM : HEPES = 8:1:1, v/v/v) which was kept on ice. Two or 5 mL of the 0.08% collagen I solution were added to each plastic dish (ø3.5 cm) quickly followed by incubation at 37°C until gels solidified with an approximate 1.2 mm thickness.

### 2.3. *Assessment of cancer cell invasion by time-lapse microscopy*

The invasion process of cancer cells on collagen gel culture model was monitored by a phase contrast time-lapse microscope ECLIPSE TE300 (Nikon, Kawasaki, Japan) housed in a temperature-controlled (37°C) $CO_2$ gassed chamber with water inside to maintain humidity. Breast or colon cancer cell lines were starved in FCS-free medium overnight, then harvested from plastic dishes by non-enzymatic separation solution (Invitrogen). Cells were seeded on the surface of the collagen gels at a concentration of $1 \times 10^5$ cells/mL in phenol red-free DMEM supplemented with 0.1% BSA and then placed in a chamber of the microscope adjusted to 37°C and a 5% $CO_2$ atmosphere. Cells remaining on the collagen gels were recorded at a 200 magnification every 1 h for 30 h. To evaluate the effect of the hormone on MCF-7 cell invasion, 10 nM β-estradiol was added to the basal medium.

The rate of cell invasion into the collagen gels was determined by plotting cell numbers remaining on the gel surface against incubation time. Data at each time point was collected from 4 to 10 independent experiments

for each condition tested. Statistical compilation was performed using StatView 5.0J (Abacus Concepts, Berkeley, CA, USA) software. The initial and maximum rates of invasion velocity were determined from the compiled data.

2.4. *Migration of cells across the collagen gel*

Collagen gels were similarly prepared on 24-well Boyden chambers with 8 μm pore size polycarbonate membranes (BD Biosciences, San Jose, CA, USA) as described above. Two hundred fifty μL of MDA-MB-231 or MCF-7 cell in DMEM with 1% FCS ($6 \times 10^5$ cells/mL) was placed on the collagen gel. After cells were incubated for 18 h in a 5% $CO_2$ atmosphere, the collagen gel on the upper surface was gently removed from the chamber. The cells were then fixed with 5% formaldehyde and stained with Diff-Quik (Dade Behring, Deerfield, IL, USA) according to the manufacturer's protocol. Cells that migrated from the collagen gel surface to the underside of the filter were counted under a microscope.

2.5. *Sectioning of collagen gels after cancer cell invasion*

Collagen gels were prepared on 8-chamber slide glasses (1 × 1 cm, Nalgen Nunc International KK, Tokyo, Japan) as described above. Two hundred fifty μL of MDA-MB-231 or MCF-7 cell suspension ($3 \times 10^5$ cells/mL) were placed on the surface of the collagen gel in phenol red-free DMEM supplemented with 0.1% BSA. After 3 h incubation at 37°C in a 5% $CO_2$ atmosphere, gels were gently washed with phosphate-buffered saline, soaked into Tissue-Tsk O.C.T. Compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C. The frozen gel was sliced using a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) set at 20 μm thickness. The sections were then placed on a slide glass, fixed with 4% formalin, and stained with hematoxylin-eosin for observation under an optical microscope.

## 3. Results

3.1. *Visualization of breast cancer cells invading collagen gels*

Invasion of breast carcinoma MDA-MB-231 and MCF-7 cells into collagen gels under the serum- and phenol red-free conditions were recorded by time-lapse microscopy. Figure 1 shows representative photo images at the indicated incubation times after the cells were placed on the collagen gel surface. Both cell types gradually disappeared from the gel surface during the time course. MDA-MB-231 cells shown in Figure 1A apparently migrated into the collagen gel faster than MCF-7 cells

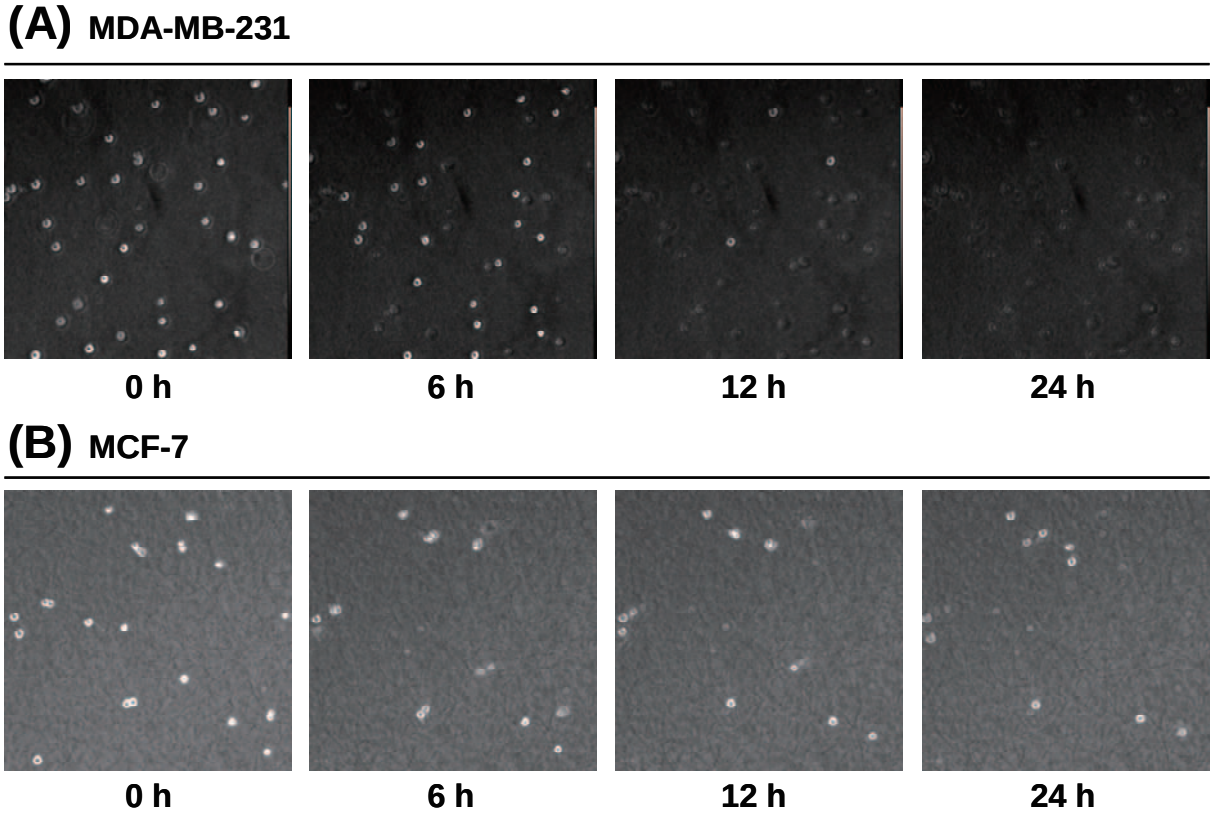


**Figure 1. Visualization of breast carcinoma cell invasion into collagen gels.** Reconstituted 0.08% collagen gel (1.2 mm-thick) was prepared in culture dish (ø3.5 cm), and then cells were inoculated on the gel at a concentration of $1 \times 10^5$ cells/mL in phenol red-free DMEM supplemented with 0.1% BSA (the basal medium). The dish was placed under a time lapse phase contrast microscope to observe the disappearance of the cells from the visible field. The photos present MDA-MB-231 **(A)** and MCF-7 **(B)** cells which remained on the gel surface at the indicated incubation time.

(Figure 1B). The traces of cells which migrated into the collagen gel were clearly marked on the surface (Figure 1).

Whether or not these cells actually moved through the collagen gels was examined using a collagen gel-coated conventional Boyden chamber as described in Materials and Methods. When the experiments were carried out under the serum- and phenol red-free condition as above, which was used throughout this study for the time-lapse recording method, none of the cells migrated across the filter (data not shown). Thus, 1% FCS was used to induce migration of cells in the Boyden chamber invasion assays. As seen in Figure 2A, a significant number of MDA-MB-231 cells (33.7 cells/60 μm$^2$) appeared on the underside of the membrane filter, and very few of the MCF-7 cells (5.7 cells/60 μm$^2$) were seen on the underside of the filter (Figure 2B) in which the 8 μm pores of the polycarbonate membrane are visible in the background. These results suggest that both the MDA-MB-231 and MCF-7 cells are capable of migrating into and through the collagen gel in the presence of 1% FCS, but that the degree of migration potential seems to be dependent on the individual characteristics of the cancer cell lines.

### 3.2. *Detection of migrated cells into collagen gels*

Time-lapse microscopic observations of the cells disappearing from the image obviously indicated the occurrence of cell migration into collagen gels under the basal conditions (Figure 1). Few cells migrated, however, from the collagen gel surface to underside of the filter even though 1% FCS was used to induce migration (Figure 2). Thus, the distance cells migrated was determined by histocytochemically examining the sectioned collagen gels. After 20 h or 18 h culture, the collagen gels into which MDA-MB-231 and MCF-7 cells migrated were cryosectioned to visualize distribution of cells inside the collagen gels. Figures 3A-3D shows representative

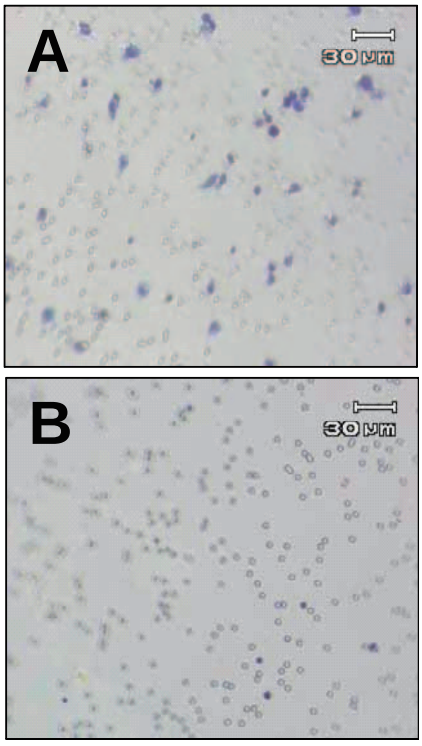


**Figure 2. Migration of breast carcinoma cells across collagen gel to underside of the membrane in a 24-well Boyden chamber.** Reconstituted collagen gel was prepared with a thickness of 1.2 mm on the membrane in chambers. MDA-MB-231 cells **(A)** or MCF-7 cells **(B)** were inoculated on the collagen gel, and then cultured for 18 h. Cells migrating to the underside of the membrane were stained with Trypan Blue and observed by microscopy (magnification ×200). Representative fields of the membrane with migrated cells are shown.

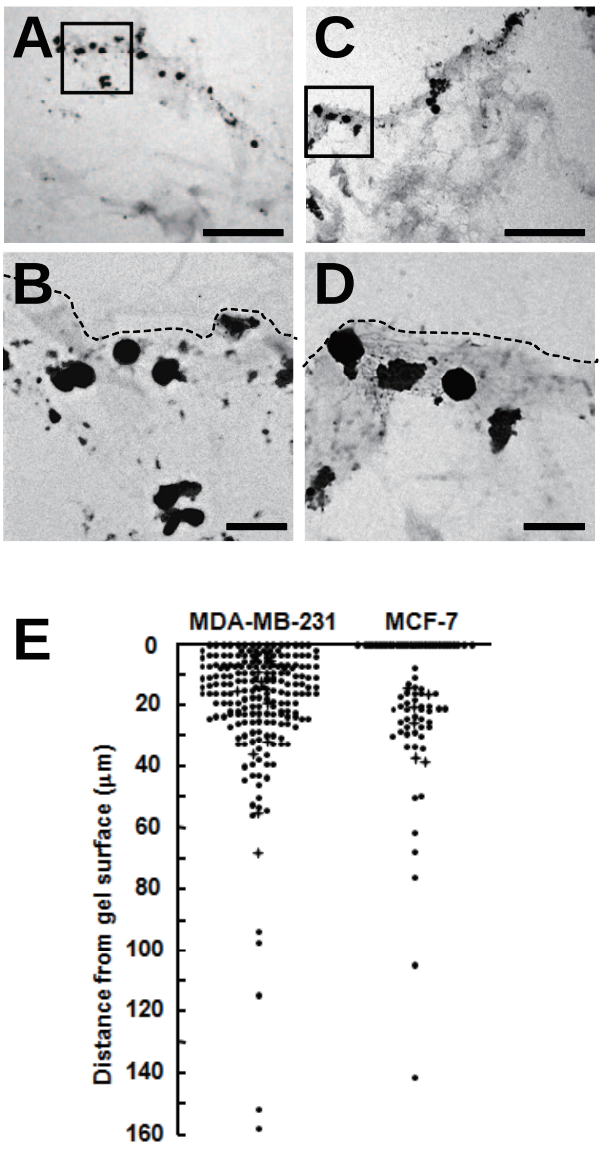


**Figure 3. Histocytochemical observation of breast carcinoma cells in reconstituted collagen gels.** MDA-MB-231 (**A** and **B**) and MCF-7 cells (**C** and **D**) in phenol red-free DMEM supplemented with 0.1% BSA (the basal medium) were incubated on collagen gels for 18 h at 37°C in 5% $CO_2$ atmosphere. The gels were frozen and sliced with a cryostat set at a 20 μm thickness. Sections were fixed and stained with hematoxylin-eosin. Areas around the gel surfaces were photographed with an optical microscope (Bars: 100 μm in **A** and **C**; 20 μm in **B** and **D**). Dotted lines in **B** and **D** represent the gel surfaces. In **E**, distribution of distances from the gel surface for each cell observed ($n$ = 214 for MDA-MB-231 cells and $n$ = 75 for MCF-7 cells) was plotted.

microscopic photos of the collagen gel sections stained with hematoxylin and eosin stain.

Migration distances of MDA-MB-231 and MCF-7 cells from the top of collagen gels measured were plotted as shown in Figure 3E. Of the 214 MDA-MB-231 cells observed, 199 (93%) cells invaded and were widely distributed in the gel. In contrast, of the 75 MCF-7 cells observed, 25 cells (33%) remained on the gel surface whereas 45 cells (60%) migrated to around 20-30 μm from the gel surface. The distances of cell migration into collagen gels are summarized in Table 1. The results suggest that cell migration certainly occurs under serum- and phenol red-free conditions, but that migration of both MDA-MB-231 and MCF-7 cells was only to 157 and 141 μm from the gel top, respectively. This result is consistent with the observation obtained by Boyden chamber assays that no cells migrated through the filter under the same conditions as used above (Figure 2).

### 3.3. *Invasion kinetics of breast and colon cancer cells*

Invasion rates of both breast and colon carcinoma cells into collagen gels are measured by counting the number of cells remaining in the same microscopic field that had been recorded by a time-lapse microscope every hour for 30 h (Figure 4). In the case of breast carcinoma cells, MDA-MB-231 cells showed a much higher disappearing rate than MCF-7 cells (Figure 4A), suggesting that MDA-MB-231 has a higher invasion potential than MCF-7 cells under the conditions used. The major difference was seen at around 25 h after cells were placed on the collagen gel, at which time nearly 100% of MDA-MB-231 cells invaded the collagen gel whereas on average 27.9 ± 4.4% ($n$ = 5) of MCF-7 cells still remained on the gel surface (Figure 4A, closed and open circles, respectively). In the case of colon carcinoma cells, LS180 cells invaded more quickly than HT29 cells (Figure 4B).

In general, cell invasion started at 1-2 h of incubation after cells were seeded on the gel. The invasion process appears to be a characteristic of each cancer cell line. For example, MDA-MB-231 breast carcinoma cells disappeared with the highest invasion rate among the four cell lines examined. In addition, all of the MDA-MB-231 cells migrated into the collagen gel before 30 h incubation whereas in the case of the other three cell lines, 22.4 ± 3.4% ($n$ = 5), 15.0 ± 2.2% ($n$ = 10), and 38.4 ± 3.1% ($n$ = 5) seeded of MCF-7, LS180, and HT-29 cells, respectively, still remained on the gel surface at the 30 h incubation time (Figure 4). Although the invasion process showed at least two phases, when the initial phase invasion rates were taken, invasion rates of 10.01 ± 2.53, 4.08 ± 1.19, 6.08 ± 1.95, and 3.48 ± 0.73%/h for MDA-MB-231, MCF-7, LS180 and HT-29 cells, respectively, were calculated. These results may reflect the malignancy of the cells.

### 3.4. *Invasion kinetics of breast and colon cancer cells*

The real-time invasion assays described above were

**Table 1. Migration distances of MDA-MB-231 and MCF-7 cells from the gel surface**

| | Migration distance (μm) | |
|---|---|---|
| | MDA-MB-231 | MCF-7 |
| Average | 31.4 | 20.1 |
| Maximum | 157 | 141 |
| 75th percentile | 24.2 | 32.9 |
| 50th percentile | 14.9 | 24.2 |
| 25th percentile | 7.1 | 18.7 |

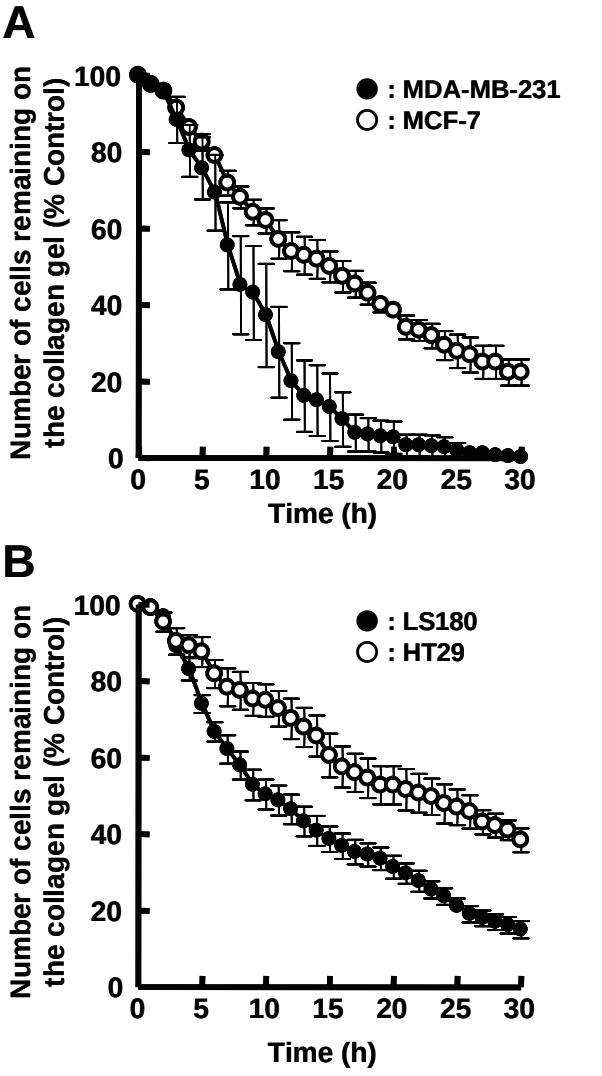


**Figure 4. Invading rates of breast and colon carcinoma cells.** Breast carcinoma MDA-MB-231 and MCF-7 cells and colon carcinoma LS180 and HT-29 cells were incubated on reconstituted collagen gels and the invasion was monitored under the time lapse microscope as described in Figure 1. The number of cells remaining on gel surface was counted and plotted against incubation time. In **(A)**, results of breast carcinoma cell invasion are shown, MDA-MB-231 (●, $n$ = 5) and MCF-7 (○, $n$ = 5); the difference is statistically significant ($p$ = 0.0015). In **(B)**, results of colon carcinoma cell invasion are shown, LS180 (●, $n$ = 10) and HT-29 (○, $n$ = 5); difference is statistically significant ($p$ = 0.0004).

under basal conditions, in which FCS and phenol red with estrogen-like effects were omitted (*9*). Although the growth of MCF-7 cells is dependent on estrogen, estrogen dependency is known to be reduced as passage numbers increase (*10*). Therefore, we examined the effects of β-estradiol on invasion of MCF-7 cells which had been cultured approximately for 10, 25, or 40 passages. As shown in Figure 5, under basal conditions, the invasion rates did not change among early, middle, and late passages of MCF-7 cells, and was on average 4.08 ± 1.19%/h ($n$ = 5) as described above (Figure 4A, open circle). The addition of 10 nM β-estradiol to the basal medium significantly facilitated the migration of MCF-7 cells with early passages (~ 10) into the collagen gels (Figure 5, closed circle) as compared to that of the cells in the absence of β-estradiol (Figure 5, open circle). Although the effect of β-estradiol on the MCF-7 cell invasion was seen with the cells in middle and late passages (Figure 5, triangle and square, respectively), the effect seemed to be less significant as the passage number increased (Figure 5).

## 4. Discussion

In this study, we established a simple procedure for real-time observation of the initial step of the cancer cell invasion process. The real-time assay method could provide not only information on invasion rates but also migratory behaviors of individual cancer cells into the collagen I gel. The Boyden chamber assay which is handy and popularly used cannot provide such detailed information although our preliminary study using Boyden chambers provided useful information that, without 1% FCS, cancer cells were not able to cross the collagen I gel under basal conditions. In contrast, this study observing the disappearance of cancer cells from the collagen I gel surface clearly demonstrated that cancer cells penetrate into the collagen I gel under basal conditions without FCS. Obviously, under the conditions used, cells did not move much on the gel surface but rather attached on the collagen gel surface and moved into the gel. Most of the cells penetrating into the gel apparently remained within 60 μm from the gel surface, which certainly indicated that cancer cells need stimuli to cross the collagen I barrier and further invade into ECM. The real-time assay system should thus allow not only direct visualization and assessment of the cancer cell invasion processes, but also study of cell invasion processes under different conditions reflecting *in vivo*.

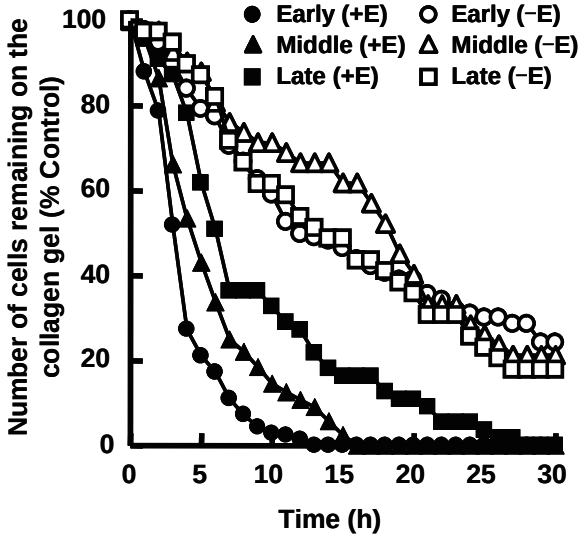


**Figure 5. Effect of β-estradiol on MCF-7 cells invasion.** MCF-7 cells with different passage numbers 10 (●, ○), 25 (▲, Δ), and 40 (■, □) were incubated on reconstituted collagen gels in the presence (●, ▲, ■: +E) or absence (○, Δ, □: −E) of 10 nM β-estradiol (E). Invasion was monitored under the time lapse microscope as described in Figure 1. The number of cells remaining on gel surface was counted and plotted against incubation time. Shown are averages of three experiments for early (−E and +E) and two experiments for middle (+E) passages whereas the result of one experiment was shown for middle (−E) and late (−E and +E) passages (statistical evaluation was not done).

Recording cell migration on or into the collagen gel was reported over three decades ago. Earlier studies using fibroblasts suggested the possible implication of this assay for cancer cell invasion (*8,11*). After rapid advances in cancer biology, several 3-dimentional culture systems have been reported for studying cancer cell invasion (*12-16)*. Of those, the real-time assay system under basal conditions described in this report is the most basic and simplest *in vitro* model for effectively assessing the invasive behaviors of individual cancer cells. The earlier study by Erkell and Schirrmacher (*12*) attempted to devise simplified systems to investigate the mechanisms of invasion based on the same concept as our study. It is the idea that since collagen I is a main component of normal tissue stroma, the ability to penetrate such a gel would be expected to reflect their metastatic potential. Their system was similar to our Boyden chamber methods with collagen I gel layered except that they counted cells which penetrated into and remained in the gel. In the Boyden chamber assays, we observed that both MCF-7 and MDA-MB-231 cancer cells did not reach the membrane under basal conditions whereas inclusion of 1% FCS in the medium induced invasion of these cancer cells to appear on the bottom side of the membrane (Figure 2).

The real-time invasion assays described here have used basal conditions, in which FCS and phenol red with estrogen-like effects were omitted. To observe effects of β-estradiol on invasion of MCF-7 cells whose growth is estrogen-dependent, three sets of MCF-7 cells from approximately 10, 25, or 40 passages were used. Although no significant difference on invasion was observed with early to late passage cells under basal conditions, the addition of 10 nM β-estradiol to the basal medium most significantly facilitated

the migration of MCF-7 cells from early passages (~ 10) into the collagen gels compared to those from middle- and late-passages. These results are consistent with a gradual loss of estrogen receptors during the progression from estrogen-dependent to -independent phenotypes (*17*).

In conclusion, the present method utilizing time-lapse microscopy is less involved and simpler than the previous methods (*14,16*), thus providing a useful system for further evaluating major components of ESM, growth factors, cytokines, and other possible microenvironment factors. Preliminary results on effects of ESM components and insulin-like growth factor-I on MCF-7 invasion have been previously presented at 12th International Congress of Endocrinology in 2004 (*18*). Further studies are in progress.

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