New Method for Separation of Subpopulations from a Heterogeneous Colon Cancer Cell Line

YOSHIHIRO TAKAYA[1], KUNIHIKO TANAKA[1], CHIASA HIRAKAWA[1], YUTAKA TAGAWA[2,3] and MASAMI NIWA[1,3]

[1]*Department of Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523;*
[2]*Department of Nursing, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8520;*
[3]*PharmaCo-Cell Company Limited, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

Abstract. *Background: To understand the heterogeneity of human colon cancers, a new method to separate cancer subpopulations was developed. Materials and Methods: Cells from a human colon cancer cell line, DLD-1, were seeded on an 8 μm pore membrane. After six hours, the cells which remained beneath the membrane as well as the cells which dropped onto the 24-well plate were collected. To clarify the differences between the two subpopulations, transepithelial electrical resistance (TEER) and immunocytochemistry were evaluated. Results: Two subpopulations, clones D and A, were separated from DLD-1 with the newly developed method. Both subpopulations showed quite different TEER values and different arrangements of cell–cell contact. In addition, the distinct subcellular localizations of claudin family proteins and zonula occludens-1 (ZO-1) were identified. Conclusion: A new separation method to isolate colon cancer subpopulations was established in which the intercellular junctions differed. This method can be considered as a helpful tool in the investigation of colon cancer heterogeneity.*

Cancer has been thought to be monoclonal for a long time even though several investigators have reported evidence of multiple tumor subpopulations within single cancer types (1). In colon cancer, heterogeneity is thought to be caused by genetic changes in proto-oncogenes, tumor suppressor genes, DNA mismatch repair genes and epigenetic changes such as DNA methylation and dysregulation of histone modification (2). A genetic model for colon cancer has been proposed in which the sequential accumulation of mutations in specific genes, including adenomatous polyposis coli (*APC*), Kirsten-ras (*K-ras*) and *p53*, drives the transition from healthy colonic epithelia through the increasingly dysplastic adenoma to colon cancer (3). Although genetic heterogeneity in colon cancer has been investigated in detail, phenotypic heterogeneity is less well studied. To investigate phenotypic heterogeneity, separation of the clones, or subpopulations, from the total cell population is needed. To isolate the monoclonal clones, both the limiting dilution method and picking up colonies with cloning cylinders are commonly used. However, the expression of specific molecules has to be examined to distinguish the clones. Using flow cytometry cell sorting and magnetic cell sorting, phenotypically distinct subpopulations of the cells can be isolated. For such occasions, specific equipment and antibodies are required.

The cultured human colon cancer cell line, DLD-1, was derived from specimens of an adenocarcinoma of human colon removed during surgery (4). DLD-1 has been identified to have two clones: clones D and A. These two clones showed different characteristics in histology, karyotype, morphology in soft agar, *in vitro* growth properties and different sensitivity to a chemotherapeutic agent (5) and irradiation (6). In this study, a new method for separation of subpopulations was developed and clones D and A were separated from the parental DLD-1 cell line. These two subpopulations showed different morphologies and transepithelial electrical resistance (TEER) values. Different interjunctional arrangements were confirmed as the cause of the TEER differences. In addition, the subcellular localizations of the claudin family and zonula oceludens-1 (ZO-1) were characterized in clones D and A. This new

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 95 8197043, Fax: +81958197044. e-mail: kunny-ta@net.nagasaki-u.ac.jp

Key Words: Separation, subpopulation, colon cancer, heterogeneity, claudin, ZO-1.

Table I. *Sequences of the primer sets and expected sizes for detecting the mRNA expressions of claudins by RT-PCR.*

Gene	Accession number	5' Primer	3' Primer	Product size (bp)
Claudin-1	NM021101	CCGTTGGCATGAAGTGTATG	CCAGTGAAGAGAGCCTGACC	208
Claudin-2	NM020384	GGGCTACATCCTAGGCCTTC	GATGTCACACTGGGTGATGC	172
Claudin-3	NM001306	CAACACCATTATCCGGGACTTC	GTAGTCCTTGCGGTCGTAGC	241
Claudin-4	NM001305	CTCCATGGGGCTACAGGTAATG	AGCAGCGAGTCGTACACCTTG	207
Claudin-5	NM003277	GAGGCGTGCTCTACCTGTTTTG	GTACTTCACGGGGAAGCTGAG	239
Claudin-6	NM021195	GATGCAGTGCAAGGTGTACG	GCCTTGGAATCCTTCTCCTC	162
Claudin-7	NM001307	TCTTGCCACCTTGGTAGCTTG	AGGACAGGAACAGGAGAGCAG	172
Claudin-8	NM199328	TGAAGGCTCACATTCTGCTG	GCCGTGGTCCATCCTAAGTAG	175
Claudin-9	NM020982	CTTCGACCGGCTTAGAACTG	GCAGAGCCAGCAGTGAGTC	216
Claudin-10	U89916	GATCATCGCCTTCATGGTCTC	GCTGACAGCAGCGATCATAAG	244
Claudin-11	NM005602	CTGGTGGACATCCTCATCCTG	CCAGCAGAATGAGCAAAACAC	190
Claudin-12	NM012129	CTCCCCATCTATCTGGGTCATC	GGTGGATGGGAGTACAATGG	201
Claudin-14	NM012130	GTCATCTCCTGCCTGCTCTC	CCTGGCCAATCTCAAACTTC	235
Claudin-15	NM014343	GGCTTCTTCATGGCAACTGTG	GGGAACTCCCAGCAGTTGTAG	173
Claudin-16	NM006580	CCAGGAATCATTGGCTCTGTG	GAACAGCTCCAGCCAAAAAG	160
Claudin-17	NM012131	AGGGCCAAAGCATACCTTCTG	CCCTTGCTTCTTTCTGTTG	246
Claudin-19	NM148960	CTCAGCGTAGTTGGCATGAAG	GAAGAACTCCTGGGTCACCAG	159
Claudin-20	NM001001346	TACTCGCTTAGGAGGGGACAG	TGCAGAAAATCATGCCAGAG	236
Claudin-23	NM194284	GTCAGCTACAGCCTGGTCCTG	GGCCGTCGCTGTAGTACTTG	217

separation method is able to isolate the phenotypically distinct subpopulations based on the differences in the formation of the tight junctions and it is therefore a useful tool in the understanding of colon cancer heterogeneity.

Materials and Methods

Cells and culture. Human colon cancer cell lines, Caco-2, DLD-1 and COLO 201 were used in the experiments. Caco-2 was purchased from the American Type Culture Collection (Manassas, VA, USA). DLD-1 and COLO 201 were donated by the First Department of Internal Medicine, Nagasaki University School of Medicine. Caco-2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml gentamycin (GM). The remaining cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 100 μg/ml GM. All media were purchased from Sigma (St. Louis, MO, USA). No evidence of mycoplasma species infection was confirmed for all cell lines using a MycoAlert™ Mycoplasma Detection Kit (Lonza, ME, USA). The cells were used for the experiments within 10 passages. All cell lines were grown in a humidified incubator at 95% air, 5% CO_2 and 37˚C.

Separation of DLD-1 subpopulations. DLD-1 cells cultured on a 35 mm dish were trypsinized and 1×10^5 cells were seeded on 12 polycarbonate support membranes of Chemotaxicell (KURABO, Osaka, Japan) with an 8 μm pore size in a 24-well cell culture cluster COSTAR 3526 (Corning Inc., NY, USA). After six hours of cultivation, each insert was removed from the culture cluster and the cells adhering to the reverse side of the membrane were detached by trypsinization and scraped with a sterile cotton swab. The detached cells were transferred to a 35 mm culture dish. The cells which had spontaneously detached from the membrane and dropped onto the bottom surface of the culture cluster were also cultured.

Transepithelial electrical resistance (TEER) measurement. TEER measurements were performed in order to evaluate the intensity of the tight junctions. For this, the cells were seeded at a density of 2×10^5 cells per well onto a Transwell® permeable support membrane (0.4 μm pore size, 1.12 cm^2 area; Corning Inc.). TEER was measured using an epithelial voltmeter (EVOM; World Precision Instruments, Sarasota, FL, USA) on days 1, 3, 5, 7, 10 and 14. The TEER for ohms×cm^2 (ohms × surface area) was calculated by subtracting the resistance of a cell-free culture insert and correcting for the surface area of the Transwell® cell culture support.

mRNA expression analysis. For total RNA extraction, 1×10^6 cells were incubated for 3 days on a 35 mm culture dish. Total RNA was isolated with the RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg of total RNA with the Reverse Transcription System (Promega, Madison, WI, USA). Polymerase chain reaction (PCR) fragments for claudins were amplified using the primer pairs showed in Table I. PCR was performed in a final volume of 25 μl containing 20 ng of template cDNA, 2 μl of dNTP mixture, 2.5 μl of 10×PCR buffer, 0.625 unit of TaKaRa Taq™ Hot Start Version polymerase (TAKARA BIO INC., Shiga, Japan) and 1 nM of each primer using PCR Express II thermal cycler (Thermo Electron Corp., Milford, MA, USA). PCR was performed with 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 59˚C for 45 seconds and extension at 72˚C for 45 seconds. The PCR products were analyzed by electrophoresis on 2.0% agarose gels and stained by ethidium bromide and were then visualized using FluorChem™ SP (Alpha Innotech, San Leandro, CA, USA). PCR for *claudin-18* was excluded due to the lack of a positive control.

Immunocytochemistry. Immunocytochemistry was performed as described elsewhere (7). The primary antibodies used in the experiments were: rabbit anti-ZO-1(Zymed, South San Francisco, CA, USA); rabbit anti-claudin-1, -2, -3, -7 and -8 (Zymed); mouse anti-claudin-4 and 5 (Zymed); goat polyclonal anti-claudin-6 (SANTA CRUZ, Santa Cruz, CA, USA) and rabbit anti-claudin-12 (Abcam, Cambridge, UK). The secondary antibodies used in the experiments were as follows: Alexa Fluor^R 488 donkey anti-mouse IgG (H+L) antibody (Molecular Probes, Carlsbad, CA, USA); Alexa Fluor^R 488 donkey anti-rabbit IgG (H+L) antibody (Molecular Probes) and FITC-conjugated anti-goat IgG antibody (Sigma). Imaging was performed using a confocal microscope equipped with an argon-krypton laser and a 63×1.4 NA plan apochromat objective lens (LSM-MicroSystem, Zeiss, Jena, Germany).

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

Results

Separation of DLD-1 subpopulations with the new method. Six hours after seeding the parental cells of DLD-1 on the Chemotaxicell 8 μm-pore, some cells passed through the membrane and remained beneath it (Figure 1, left, white star). Other cells which passed through the membrane dropped onto the culture cluster (Figure 1, left, black star).

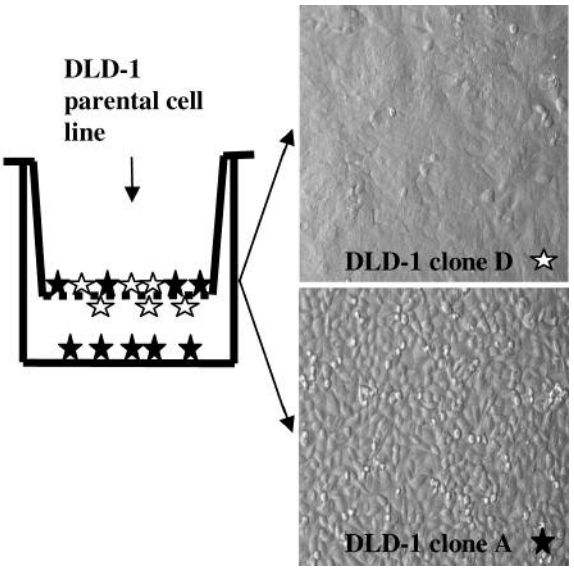

Figure 1. *Separation method of clones D and A from the DLD-1 parental cell line and phase contrast imaging of the 2 clones. A total of 1×10^5 cells were seeded on an 8 μm pore membrane. Clone D (left, white star) was subcloned from the cells adhering to the reverse side of the membrane, and clone A (left, black star) from the cells which had detached from the membrane and dropped onto the bottom. Clone D (upper right) and clone A (lower right) showed different morphology. Original magnification was ×200.*

The cells which remained beneath the membrane had a flat morphology and were tightly attached to each other (Figure 1, upper right), whereas the cells which had detached and dropped onto the bottom appeared swollen and were separated from each other (Figure 1, lower right). Based on their morphological characteristics, the cells which remained beneath the membrane were identified as clone D, whereas the cells which had dropped onto the bottom were identified as clone A. It is noted that the morphological differences were not influenced by the confluency of the cells.

TEER measurements. To investigate differences between the cell-cell contact, TEER measurements of clones D and A were performed. Interestingly, the TEERs between clones D and A showed significant differences (Figure 2). The TEER of clone D was 147±1.4 ohms cm^2 on day 14, whereas that of clone A was 12±0 ohms cm^2 on the same day. These data indicate that the tight junctions of clone D are much stronger than those of clone A.

mRNA expression of the claudin family. Reverse transcription (RT)-PCR was performed to examine the expression patterns of claudin family genes in Caco-2, COLO 201 and 2 DLD-1 clones. Claudin-1 to -7, -10, -11, -12, -15, -17, -19, -20 and -23 were expressed in all cell lines ubiquitously (Figure 3). However, claudin-9 and -14 were not expressed in Caco-2 and

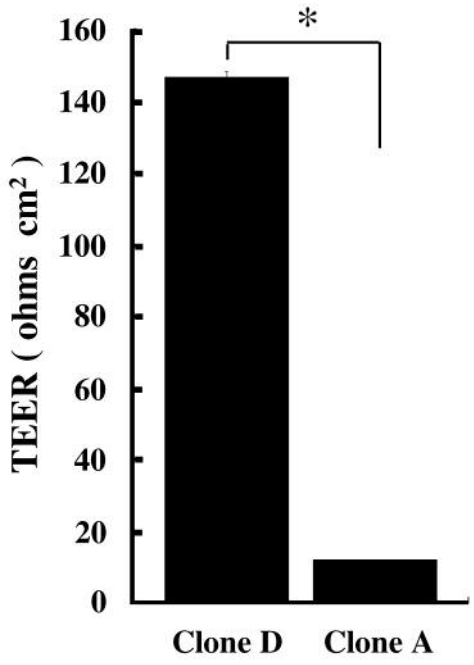

Figure 2. *The TEER of the DLD-1 clones. A total of 2×10^5 cells were seeded on a 0.4 μm pore membrane, and the TEER was measured on days 1, 3, 5, 7, 10 and 14. The TEERs on day 14 are shown as ohms cm^2. The data are presented as the means±S.E. of 3 replicates from 2 independent experiments. *p<0.01, comparison between clone D and A.*

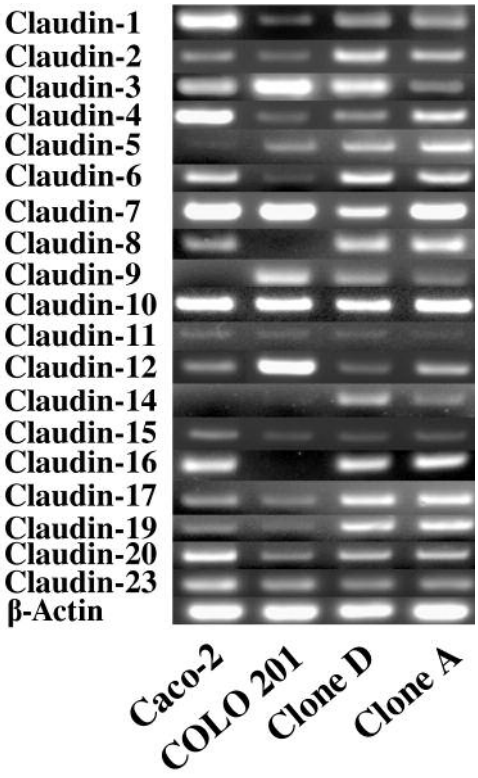

Figure 3. *The expressions of claudin family mRNA in human colon cancer cell lines and DLD-1 clones. First strand cDNAs were synthesized from 1 μg total RNA from cancer cells and amplified by PCR using specific primers for claudins. The PCR products were qualitatively analyzed on 2.0% agarose gel.*

claudin-8, -14 and -16 were not expressed in COLO 201. Although RT-PCR showed quantitative differences of mRNA expressions in claudin-2, 3, 4, 7, 12 and 14 between clone D and A, no significant qualitative differences were clarified.

Immunocytochemistry analysis. To clarify the subcellular localizations of tight junctional molecules, immunocytochemistry of the two clones was evaluated. Firstly, the differences in cell-cell contact between clones D and A were confirmed. Clone D cells were connected to adjacent cells tightly, whereas the intercellular junctions of clone A cells were loose and had openings, even after the cells formed a complete monolayer (Figure 4). These differences in cell-cell contact are thought to be responsible for the differences of the TEERs between clones D and A. In clone D, claudin-1, -2, -3, -4, -5 and -12 were observed in all of the membranous, cytoplasmic and nuclear regions. Claudin-7 was expressed dominantly in a continuous membranous pattern and to some extent in the cytoplasmic region, although it was not expressed in the nuclear region. Claudin-6 and -8 were mainly expressed in the nuclear region and in the cytoplasmic region to some extent. However, these two claudin proteins were not expressed in the membranous region. In clone A, claudin-1, -2, -3, -5, -6, -8 and -12 were expressed in the nuclear and cytoplasmic regions in a dot-like manner. Claudin-4 and -7 were localized in the perinuclear and cytoplasmic region rather than in the nuclear region. Claudin-1, -3, -4, -5 and -12 were also weakly expressed in the membranous region in a dot-like manner. In clone D, ZO-1 was expressed in the membranous region with a linear pattern. In contrast, ZO-1 was expressed in the perinuclear, cytoplasmic and possibly membranous region in a dot-like manner in clone A. In clones D and A, ZO-1 was not expressed in the nuclear region.

Discussion

A new method for separation of subpopulations from a cancer cell line was developed. DLD-1 was selected as a sample because it has been reported to show heterogeneity and contain two clones: clones D and A. Originally, these two clones were subcloned in soft agar based on the differences in their morphology (8, 9). In the present method, clone D passed through the 8 μm pore membrane and remained adhered to the reverse side of the membrane. Clone A passed through the membrane, dropped and then

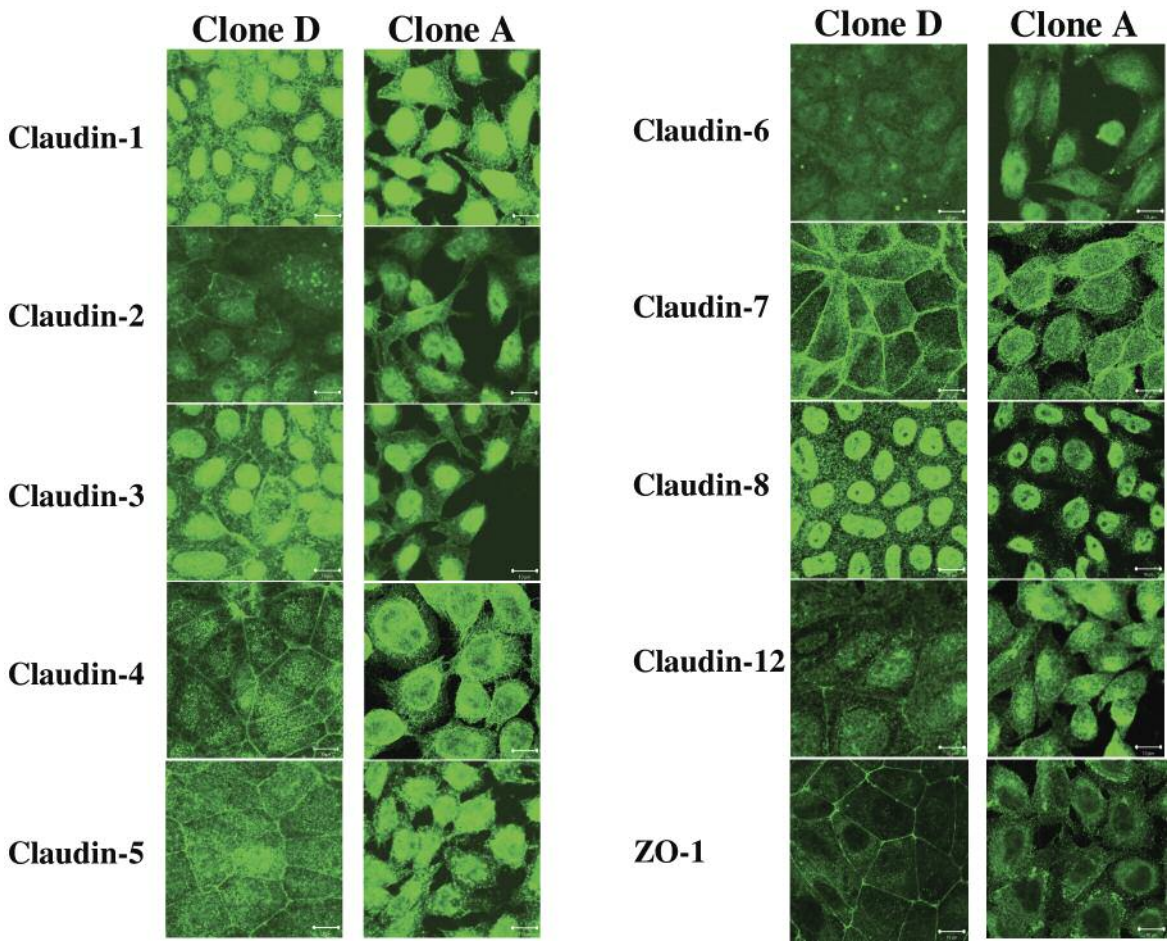

Figure 4. *The subcellular localization analyses of claudin family and ZO-1 in DLD-1 clones by immunocytochemistry. Cells were plated and maintained under confluent conditions. After fixation and permeabilization, the cells were labeled with antibody and fluorescence signals were detected under confocal fluorescence microscopy. The scale bar represents 10 μm.*

continued to grow on the culture cluster. It is clear that clone D is more adhesive to the membrane than clone A, and therefore the migration activity of clone D is weaker than that of clone A. The more invasive potential of clone A over that of clone D was also confirmed using an invasion assay and wound healing assay (data not shown).

TEER measurements were performed to clarify the differences of the tight junction (TJ) structure between the two clones. Clone A showed very low TEER values, whereas clone D showed high TEER values. This is the first report in which two clones originating from the same parental cell line showed different TEERs. The data indicated that clone A has "leaky" TJ and clone D has "tight" TJ. Colon cancer cells are originated from colonic epithelia colonocytes. Normal epithelial cells with high TEER maintain cell polarity and control the permeability of the substrates with rigid TJs (10). The deformation or change of the expression pattern in the TJ results in a loss of cell polarity in several types of cancer (11, 12) and increased permeability of the TJ is associated with the development of colon cancer (13). These data suggest that some of the characteristics of normal colonic epithelia with rigid TJ structure and cell-cell contact remain in clone D but not in clone A. Although clone D seems to be similar to the normal colonic epithelia before oncogenic transformation, it is unclear whether clone D was transformed from normal colonic epithelia and clone A is derived from clone D, or that these two clones derived from the same origin, such as a cancer stem cell, independently. Further experiments are therefore needed to clarify the origin of these two clones.

An immunocytochemical analysis showed that at least claudin-2, -3, -4, -5, -7 and -12 played a role in the formation of TJ at the cell boundaries and ZO-1 was lined at the membrane sites in clone D. In contrast, most of the claudins could not be expressed functionally at the membrane sites in clone A. The ZO-1 expression in the

membranous region in clone A was very low and was in a dot-like or discontinuous pattern. Umeda *et al*. reported that ZO-1 and ZO-2 located in the membranous region were essential for claudins to polymerize and construct TJ strands (14). It is likely that the lack of ZO-1 accumulation in the membranous region may result in the lack of TJ formation by polymerized claudins in clone A.

In the present method, the difference between clones D and A was whether or not the cells dropped from the reverse side of the 8 μm membrane. The 8 μm pore membrane is commonly used for migration assays (15) and Matrigel invasion assays (16). In these assays, the cells which pass through the membrane and remained adhering to the reverse side of it are counted, and the cells which pass through the membrane and drop onto the bottom are usually ignored or thought to be less important. Okada *et al*. reported that when HT1080 human fibrosarcoma cells were seeded in a chamber on the endothelial monolayer on a laminin-coated 8 μm pore membrane and incubated for 24 h, these cells migrated to the lower chamber (17). Based on these observations, focus was shifted onto the subpopulation of dropped cells and a new method was developed. This method allows the distinction between the characteristically and morphologically different subpopulations from cancer cell lines contrary to the limiting dilution method and subcloning with cloning cylinders. The selectivity of the resultant subpopulations using the method presented here depends on the detachment from the membrane and the ability of anchor-independent growth (anoikis resistance). These properties play a key role in obtaining the phenotypically distinct subpopulations in this method. Further studies on the use of this separation method with other cancer cell lines and clinical samples from patients are currently underway in our laboratory. In conclusion, a new separation method for colon cancer subpopulations in which the intercellular junctions differ was developed. This method is both easy to perform as well as a useful tool to investigate the heterogeneity of colon cancer.

Acknowledgements

We would like to thank the First Department of Internal Medicine, Nagasaki University School of Medicine for the gift of DLD-1 and COLO 201. This study was supported in part by a grant from the New Industry Creative-Type Technology R&D Promotion Programs from the Ministry of Economy, Trade and Industry (2005, 2006).

References

1 Heppner GH: Tumor heterogeneity. Cancer Res *44*: 2259-2265, 1984.

2 Wong JJ, Hawkins NJ and Ward RL: Colorectal cancer: a model for epigenetic tumorigenesis. Gut *56*: 140-148, 2007.

3 Smith G, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ and Wolf CR: Mutations in *APC*, *Kirsten-ras*, and *p53* – Alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci USA *99*: 9433-9438, 2002.

4 Dexter DL, Barbosa JA and Calabresi P: *N,N*-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. Cancer Res *39*: 1020-1025, 1979.

5 Mellor HR, Ferguson DJ and Callaghan R: A model of quiescent tumour microregions for evaluating multicellular resistance to chemotherapeutic drugs. Br J Cancer *93*: 302-309, 2005.

6 Leith JT, Dexter DL, DeWyngaert JK, Zeman EM, Chu MY, Calabresi P and Glicksman AS: Differential responses to x-irradiation of subpopulations of two heterogeneous human carcinomas *in vitro*. Cancer Res *42*: 2556-2561, 1982.

7 Tanaka K, Xu W, Zhou F and You G: Role of glycosylation in the organic anion transporter OAT1. J Biol Chem *279*: 14961-14966, 2004.

8 Dexter DL and Hager JC: Maturation-induction tumor cells using a human colon cancer model. Cancer *45*: 1178-1184, 1980.

9 Dexter DL, Spremulli EN, Fligiel Z, Barbosa JA, Vogel R, Van Voorhees A and Calabresi P: Heterogeneity of cancer cells from single human colon carcinoma. Am J Med *71*: 949-956, 1981.

10 Burgel N, Bojarski C, Mankertz J, Zeitz M, Fromm M and Schulzke JD: Mechanisms of diarrhea in collagenous colitis. Gastroenterology *123*: 433-443, 2002.

11 Naishiro Y, Yamada T, Takaoka AS, Hayashi R, Hasegawa F, Imai K and Hirohashi S: Restoration of epithelial cell polarity in a colorectal cancer cell line by suppression of beta-catenin/T-cell factor 4-mediated gene transactivation. Cancer Res *61*: 2751-2758, 2001.

12 Klezovitch O, Fernandez TE, Tapscott SJ and Vasioukhin V: Loss of cell polarity causes severe brain dysplasia in Lgl1 knockout mice. Genes Dev *18*: 559-571, 2004.

13 Soler AP, Miller RD, Laughlin KV, Carp NZ, Klurfeld DM and Mullin JM: Increased tight junctional permeability is associated with the development of colon cancer. Carcinogenesis *20*: 1425-1431, 1999.

14 Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M and Tsukita S: ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell *126*: 741-754, 2006.

15 Romualdez AG Jr and Ward PA: A unique complement-derived chemotactic factor for tumor cells. Proc Natl Acad Sci USA *72*: 4128-4132, 1975.

16 Hendrix MJ, Seftor EA, Seftor RE and Fidler IJ: A simple quantitative assay for studying the invasive potential of high and low human metastatic variants. Cancer Lett *38*: 137-147, 1987.

17 Okada T, Okuno H and Mitsui Y: A novel *in vitro* assay system for transendothelial tumor cell invasion: significance of E-selectin and alpha 3 integrin in the transendothelial invasion by HT1080 fibrosarcoma cells. Clin Exp Metastasis *12*: 305-314, 1994.

Received July 25, 2008
Revised October 3, 2008
Accepted October 15, 2008