Anoikis-resistant MDCK cells carrying susceptibilities to TNF- α and verotoxin that are suitable for influenza virus cultivation

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Abstract Madin-Darby canine kidney (MDCK) cells were originally anchorage-dependent epithelial cells. Here, we have isolated a novel MDCK-derived cell population, termed 6 M-4, by means of culturing MDCK cells in suspension for nearly 6 months in the presence of *Streptomyces griseus* metalloendopeptidase (MEP). The isolated cells showed unique proliferation characteristics, which differed from parental MDCK cells. They proliferated adherently on a polystyrene matrix, but proliferated non-adherently both in the presence of MEP and on a non-adhesive matrix coated with poly 2-methacryloyloxyethyl phosphorylcholine (MPC). The 6 M-4 cells consisted of at least two cell types. One type, termed 6 M-4-TR7, would not

grow in soft agar and showed a novel phenotype in that the cells were susceptible to both TNF- α and verotoxin 1 (VT1). In addition, the isolated adhesion-independent cells sustained epithelial traits of parental MDCK cells. We further show that these MDCK-derivative cells are suitable for influenza virus cultivation. Hemagglutination (HA) titers of influenzaviruses A and B were increased in the suspension culture of 6 M-4-TR7 cells supplemented with the MEP in comparison to adherently growing cells in the presence of trypsin.

Keywords MDCK · Anoikis-resistance · Cell adherence · TNF- α · Verotoxin · Influenza virus

Abbreviations

MEM	minimum essential medium
MEP	<i>Streptomyces griseus</i> metalloendopeptidase
MPC	poly 2-methacryloyloxyethyl phosphorylcholine
rTNF- α	recombinant tumor necrosis factor α
VT1	verotoxin 1

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Introduction

MDCK is an anchorage-dependent epithelial cell line derived from canine kidney and shows anoikis

(Ruoslahti et al. 1994). A suspension culture of this cell line has been devised for the large-scale production of the influenza virus by introducing solid carriers to the medium (Tree et al. 2001; Merten et al. 1996) or by culturing the cells with shaking. The latter is a typical culture method recommended for the MDCK cells to adapt to growth in a non-adherent suspension (Sinacore et al. 2000; Valley et al. 2006). However, the mechanisms underlying the transition of the proliferation status of this cell type from matrix-adherent to suspension are not fully understood and are under investigation in terms of the interplay between integrin signaling and apoptosis signaling pathways including focal adhesion kinase (FAK) and Rho family GTPases (Frisch et al. 1996; Cheng et al. 2004). In these cases, the anoikis-resistant anchorage-independent growth of the MDCK cells was accompanied by a significant increase in the tumorigenic potential, though the causal relationship among them is not clear. On the other hand, recent studies have revealed the participation of matrix metalloproteinases (MMPs) and their corresponding tissue inhibitors (TIMPs) in cell survival signaling (Bian et al. 1996; Liu et al. 2003; Salge et al. 2001; Hotary et al. 2003). Hotary et al. (2003) reported that membrane type-1 matrix metalloproteinase conferred both nontumorigenic and tumorigenic cell lines with a three-dimensional extra cellular matrix growth advantage. Successful conditions of suspension culture in the presence of bacterial neutral protease from *Bacillus polymyxa*, dispase, for several cell lines were already reported (Nagata and Matusda 1986). The possibility of suspension culture of MDCK was also mentioned in their report. From these points of view, we attempted to use metalloendopeptidase (termed MEP) from *Streptomyces griseus* for the selection of cells with altered pathways of survival and/or death signaling, e.g. those relating to anoikis. This approach enabled us to obtain anoikis-resistant MDCK cells. The isolated anoikis-resistant cells showed sensitivities to TNF- α and verotoxin (VT, also called Shiga toxin, Stx), a phenotype that the parental MDCK cells did not carry. In addition, the isolated anoikis-resistant MDCK cells sustained epithelial traits and did not show malignant transformation. Further, we show that the isolated

anoikis-resistant MDCK cells are useful for the cultivation of influenza viruses as compared to the original parental cells.

Materials and methods

Proteases

Metalloendopeptidase (MEP) (Fujisaki 1996) is purified from the fermentation broth of a strain of *S. griseus* through processing on size exclusion, ion exchange and ligand-fixed affinity column chromatography (KAKEN Pharmaceutical Co., Ltd. Tokyo, Japan). A typical preparation of MEP shows 96% purity on reverse phase HPLC analysis and a single protein band on SDS-PAGE analysis. The molecular weight is about 38,000 (on SDS-PAGE analysis). The stable pH range is between 3.0 and 9.5. The isoelectric point (pI) is 4.9. MEP has one atom Zn in a molecule. The cleavage sites are at amide bonds of carbobenzoxy-Ala-Leu-NH₂, carbobenzoxy-Gly-Leu-NH₂, carbobenzoxy-Gly-Phe-NH₂ and at *p*-nitroanilide (*p*NA) bonds of Pyr-Phe-Leu-*p*NA, carbobenzoxy-Ala-Ala-Leu-*p*NA and carbobenzoxy-Gly-Gly-Leu-*p*NA. Amino acid sequences of MEP have been reported (Tsuyuki et al. 1991; Kojima et al. 1998; Kato 2002). Dispase (grade II), a bacterial neutral proteinase, was purchased (Invitrogen, Carlsbad, CA, USA.) and added by the concentration of 0.6 U/ml in PBS or in culture media to detach the monolayer cells from the floor of culture flask.

Cells and culture-ware

MDCK cells were purchased from ATCC (NBL-2, CCL 34) and cultured in polystyrene culture flasks (Nalge Nunc International, Rochester, NY, USA, No136196) in Minimum Essential Medium (MEM) (Nissui Pharm. Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (MEM-10) (Gibco, Rockville, MD, USA) and 2 mM L-glutamine (Kanto kagaku Co., Tokyo, Japan), at 37°C under 5% CO₂. Cells grown to confluence on day 7 were detached using 0.25% trypsin-EDTA (Difco Lab, MI, USA) in phosphate-buffered saline without Ca²⁺ nor Mg²⁺ (PBS, pH 7.4), and transferred to

fresh medium. The culture flask and the 96-well cell culture plate coated with 2-methacryloyloxyethyl phosphorylcholine (MPC) co-polymer (Low Cell Binding Flask, Custome Made) were purchased from Nunc A/S (Roskilde, Kamstrupvej, Denmark). For immunocytochemical staining, cells were grown on LAB-TEK II Chamber slides (Nalge Nunc Co.). Vero cells were purchased from RIKEN BioResource Center (WRC002, Tsukuba, Japan) and cultured in MEM-10.

Isolation of MEP-resistant MDCK Cells

MDCK cells were cultured for 6 months in the presence of 50 µg/ml MEP by exchanging medium twice a week. The cells that survived were recovered and maintained as adherent cells in MEM-10 without MEP. Colony selection was carried out by the dilution method on a 96 well polystyrene plate and by proliferation activities in soft agar. For the latter selection, cells were suspended in 15 ml of 0.3% Difco™ Agar Noble (BD, Sparks, MD, USA) in complete growth medium. This cell suspension was allowed to solidify at room temperature on top of 10 ml 0.5% agar in 10 cm-diameter dishes. On day 10, grown cells or colonies were picked out and cultured adherently in 96-well plates.

Nested PCR for mycoplasma detection

Two micro liters of culture medium was obtained from the MDCK and the derived cells 7 days after the passage and subjected to the first PCR in 20 µl reaction mixture using PCR Mycoplasma Detection Kit (TaKaRa Biotechnology, Tokyo, Japan) and AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). For the first PCR, F1 primer: 5' ACACCATGGGA GCTGGTAAT and R1 primer: 5' CTTCWTCGACTTYCA-GACCCA AGGCAT, and for the nested PCR, F2 primer: 5' GTTCTTTGAAACTGAAT and R2 primer: GCATCC ACCAWAWACTCT were used. Ethidium bromide staining was done after gel electrophoresis of the first and nested PCR products aligned with the positive control. Possible mycoplasma contamination was also tested in another laboratory.

Measurement of aggregation size

The confluent monolayer culture of the parental MDCK cells and 6 M-4 cells were dispersed with trypsin, resuspended in the MEM-10 and transferred to MPC-coated flasks separately. Then the cells were observed after 3 and 7 days of culture. The maximal diameter of each fifty aggregates selected arbitrarily in one field (magnification of $\times 200$ or $\times 400$) was measured by the use of AxioVision (Carl Zeiss Co. Ltd, Jena, Germany).

Scanning and transmission electron microscopy

Floating cells were collected by centrifugation, re-suspended in PBS, and centrifuged again. The pellet was fixed for 24 h with 2% glutaraldehyde and post-fixed with 1% osmium oxide solution for 2 h. For scanning electron microscopy, the cells dehydrated with graded ethanol and 2-methyl 2-propanol were put on the sample disk and coated with osmium using osmium plasma coater (MW-PC30). Observation was performed with a Hitachi S-4700 scanning electron microscope. For transmission electron microscopy, after dehydration with graded ethanol and substitution with n-butyl glycidyl ether (Nissin EM Co., Tokyo, Japan), the cells were embedded on TAAB Epon 812 (TAAB, Berkshire, England). Ultrathin sections were stained with uranyl acetate for 30 min, with lead citrate for 5 min, and subjected to transmission electron microscopy using a Hitachi H-7100 (Hitachi Co., Tokyo, Japan).

Evaluation of cell viability and growth

The viability of cells was checked with calcein AM and ethidium homodimer-1 (Molecular Probes, Inc., Eugen, OR, USA). Stock solutions of calcein AM (1 mM in dimethylsulfoxide) and ethidium homodimer-1 (1 mM in water) were added to the recovered floating cells in PBS to a final concentration of 25 µM and 1 µM, respectively. After incubation for 30 min at 37°C, the cells were washed with PBS and their morphological appearances were examined using fluorescent microscopy, a BX50 microscope equipped

with a DP50 digital camera attached (Olympus Co., Tokyo Japan).

Respiratory activities of the cells were measured using an alamarBlue™ (Trek diagnostic System Inc., Cleveland, OH, USA). Cells grown to 90% confluence were treated with trypsin, and 1×10^4 cells in 100 μ l were seeded in a 96-well culture plate. At a fixed time each day, 10 μ l of alamarBlue™ reagent was added to the wells, cultured for 6 h and the fluorescence of the reduced alamarBlue™ was monitored at a 530 nm excitation wavelength and 590 nm emission wavelength using a Luminescence spectrometer LS 50B (Perkin-Elmer Ltd., Buckinghamshire, UK).

Incorporation of ^3H -thymidine (Amersham Biosciences Corp. Piscataway, NJ, USA) into high molecular weight DNA in the cells was assayed essentially according to the procedure described by Shevach (1996). Cells in the 96-well culture plate were labeled with ^3H -thymidine. After addition of Triton X to a final concentration of 0.1%, cells were harvested with a semi-automated cell harvesting apparatus and counted for radioactivities using β counter LSC-1000 (ALOKA Co., Ltd, Tokyo, Japan).

Apoptosis assay

Apoptotic cells were stained with FITC-labeled Annexin V and propidium iodide using an Apoptosis Detection Kit (Trevigen, Inc. Gaithersburg, MD, USA). Floating and adherent cells were recovered by centrifugation after incubation with rTNF- α for 6 h. To the cell pellet (ca 1×10^6 cells), 200 μ l of the binding buffer and 10 μ l each of Annexin V and propidium iodide (PI) solutions were added according to the manufacture's protocol. Apoptotic cell fractions which displayed Annexin V-positivity were analyzed using a flow cytometer (FACS Calibur™, BD Bioscience, San Jose, CA, USA). To confirm the apoptotic DNA fragmentation after TNF- α treatment, the condition of cell cycle analysis was applied to cells fixed with 70% ethanol and stained with PI (50 μ g/ml).

Immunocytochemical detection

Cells grown on the LAB-TEK II Chamber slide (Nalge Nunc Co.) were washed with PBS, fixed

with cold methanol for 15 min, and labeled with anti-E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), anti-vimentin (Sigma-Aldrich, Inc.), anti-cytokeratin (Progen Biotechnik GmbH, Heidelberg, Germany) antibodies for four hours at room temperature. For binding anti-GB3/CD77 (UK-Serotec Ltd., Oxford, UK) antibody, cells were fixed with 2% paraformaldehyde (Merk Co., Darmstadt, Germany) in PBS for 15 min. FITC labeled anti-mouse IgG (Immuno-tech Coulter Co., Marseille, France) and anti-rabbit IgG (BD Biosciences Co) anti-rat IgM (UK-Serotec Ltd) antibodies were used to detect the antigen-bound antibodies using fluorescent microscopy as described above. The actin filament was stained with rhodamine labeled phalloidin (Molecular Probes, Inc., Eugen, OR, USA) for the cells fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X.

Susceptibilities to TNF- α and verotoxin 1

Cells grown to near confluency were recovered by trypsinization, and 1×10^4 cells in a 100 μ l suspension were seeded in a 96-well cell culture plate. Then, serially diluted solutions of verotoxin 1 (VT1, Denka Seiken Co., Tokyo, Japan) or recombinant mouse TNF- α (rmTNF, Genzyme Co., MA, USA) were added and incubated for 3 days. After incubation, the culture medium containing the floating dead cells was removed and 100 μ l of a fresh medium containing 5 μ l of alamarBlue™ was added. The redox activities of the adherent cells were monitored as described in the "evaluation of cell viability and growth" section.

Thin layer chromatography of neutral glycolipids

Vero, MDCK, 6 M-4 and 6 M-4-TR-7 cells grown in a 25 cm² polystyrene culture flask were recovered by trypsinization and centrifugation. The lipid fraction was extracted with 5 ml of a chloroform/methanol (2:1) mixture and concentrated to dryness. An aliquot of samples dissolved in chloroform/methanol (2:1) was developed on a silica gel 60 F254 plate (Merck KGaA, Darmstadt, Germany) using a chloroform/methanol/water

(65:25:4) system with the TLC standard of neutral glycolipids (IsoSep AB, Tullinge, Sweden). Orcinol ferric chloride reagent (SIGMA Chem. Co) was sprayed and the plates were kept at 100°C for 15 min for the detection.

Inoculation of influenza virus

Strains of influenza virus A/New Caledonia/20/1999/H1N1, A/Panama/2007/1999/H3N2, B/Shandong/7/97 were kindly provided by Dr. Odagiri, National Institute of Infectious Diseases, Tokyo. Influenza A/Morioka/O-68N/2000/H3 (Morioka City, Japan, N type is not defined) strain was isolated at Research Institute for Environmental Sciences and Public Health of Iwate. The virus stock solution was prepared with MDCK cells. Suspension and adhesion cultures of MDCK cells and their derivatives were inoculated with an aliquot of influenza virus at the multiplicity of infection (MOI) of 0.002 or 0.001. For the suspension culture condition, cells grown adherently to near confluency (day 5) were recovered by trypsinization and transferred to MPC flask and kept further two days in the MEM-10. The MEP (25 µg/ml) was added to the suspension culture just prior to the virus inoculation. The growth medium (MEM-10) was replaced by the MEM (without FCS) supplemented with 2 U/ml of trypsin (Mochida Pharm. Co., Tokyo, Japan) just prior to the virus inoculation to the adherent cells in the polystyrene flask and to another suspension cells in the MPC flask. The MEP-containing medium was supplemented with 10% FCS throughout the culture. During the course of culture, the virus suspension was recovered from the culture medium by centrifugation at $1,500 \times g$ for 15 min. The virus antigen titer was determined by hemagglutination (HA) test using chicken red cells (Nippon Biotest Lab. Inc., Tokyo, Japan). To the 25 µl of the serially diluted virus solutions in a U-shape 96 well plate (Nalge Nunc), 25 µl of PBS and 50 µl of the red cell suspension (0.5% volume in PBS) were added. After 90 min the endpoint of hemagglutination was evaluated by direct observation. The infectivity of the recovered supernatant was confirmed as follows. To confirm the infectivity of recovered virus, one hundred microliters of the serially

diluted virus was used to infect MDCK cells cultured in rolling test tubes (four tubes for each sample) containing 1 ml of MEM supplemented with 2 U/ml of trypsin. The median tissue culture infectious dose (TCID₅₀) of the virus suspension was evaluated by direct observation of the development of a cytopathic effect (CPE) for 10 days.

Results

MEP detaches adherent MDCK cells in a way different from trypsin and Dispace

Trypsin has been widely used to detach cultured cells from culture-ware matrices. As shown in Fig. 1, MEP caused adherent MDCK cells grown in polystyrene dishes to become single cells in the same way as trypsin did in the absence of FCS (Fig. 1A). However, in the presence of FCS, MEP dislodged adherent cells as a sheet (Fig. 1B). When dispace was used instead of MEP, adherent MDCK cells became detached as a sheet either in the presence or absence of FCS (Fig. 1C and D), as has been reported by Nagata et al. (1986). Thus, MEP exhibited a unique proteolytic property against MDCK cells that differ from dispace.

Isolation of MEP-resistant floating cells

As described above, MEP caused adherent MDCK cells to become non-adherent. Then, MDCK cells were cultured for about 6 months under the continuous exposure to 50 µg/ml MEP, with passages two times a week into fresh medium. After that, a floating cell population of MDCK cells that survived in the presence of MEP was isolated, and designated as 6 M-4 cells. Figure 2 shows the morphological appearance of the isolated 6 M-4 cells. The 6 M-4 cells proliferated in the form of floating aggregates in non-adhesive MPC-matrix-coated culture ware or in the presence of MEP (Fig. 2A and C), whereas the parental MDCK cells died in the non-adhesive culture ware or in the presence of MEP (Fig. 2B and D). The 6 M-4 cells grew adherently in the MEM-10 medium without MEP in the normal polystyrene culture ware, similar to parental MDCK cells (Fig. 2E and F). Staining

Fig. 1 MEP and dispase act differently on adherent MDCK cells. MDCK cells grown attached on polystyrene dishes were washed and treated with 50 μ g/ml MEP in PBS (A), 50 μ g/ml MEP in MEM containing 10% FCS (MEM-10)(B), 0.6 U/ml of dispase in PBS (C), and 0.6 U/ml of dispase in MEM containing 10% FCS (D)

with calcein-AM and ethidium homodimer revealed that the floating 6 M-4 cells in the presence of MEP were ethidium-homodimer-negative, indicating that these cells are viable (Fig. 2G). As expected, a large fraction of the parental MDCK population cultured with MEP showed positive signals of ethidium homodimer (Fig. 2H). Scanning and transmission electron microscopy showed that the clusters of suspension cells were separated from each other by intercellular adhesion structures (Fig. 2I and J).

Sizes of the cell aggregates

Most of the parental MDCK cells were dead on the 3rd day of the culture so that the sizes were homogeneously small (Fig. 3A), but larger clusters were observed in the 6 M4 populations on the 3rd and 7th day, especially on the 7th day, which can be cultured for longer period (Fig. 3B).

MEP-resistant 6M-4 cells are anoikis-resistant

To see anoikis-resistant survival of the 6 M-4 cells, inversion of membrane phosphatidylserine, a typical phenomenon of apoptosis, after detachment of the cells was examined using FITC-

labeled Annexin V that specifically binds to it. The 6 M-4 cells cultured for 6 h in MPC- or polystyrene-coated dishes were not stained with FITC-Annexin V (Fig. 4A and C). In contrast, a larger fraction of the parental MDCK cells grown on the MPC matrix showed Annexin V-positive signals as compared to the cells in polystyrene matrix dishes (compare Fig. 4B and D), indicating inversion of cell membrane of the parental MDCK cells when they were put under non-adherent conditions. Both 6 M-4 and parental MDCK cells just after detachment from the polystyrene dishes by trypsin did not bind to FITC Annexin V (Fig. 4E and F). Figure 4G and H are control experiments of FACS analysis without binding of Annexin V. These results indicate that the 6 M-4 cells proliferate in an anoikis-resistant way.

The anoikis-resistant survival of the 6 M-4 cells was further monitored by respiratory activity and DNA synthesis activity. As shown in Fig. 5, respiratory activities of the 6 M-4 cells suspension-cultured in the presence of MEP were not affected but rather stimulated (5A), whereas, the activities of parental MDCK cells were sensitive to the MEP (5B). As to assess DNA synthesis activity, incorporation of ^3H -thymidine into

Fig. 2 Viability of 6 M-4 cells cultured adherently and non-adherently. 6 M-4 cells (A, C, E and G) and parental MDCK cells (B, D, F and H) were cultured for 7 days in MPC-coated dishes (A, B), in normal polystyrene dishes in the presence (C, D) or absence (E, F) of 50 $\mu\text{g}/\text{ml}$ MEP. G and H show 6 M-4 and MDCK cells, respectively, after staining with calcein-AM (live cells) and ethidium homodimer (dead cells). Scanning and transmission electron microscopy of floating 6 M-4 cells are shown (I, J) (J2 is a higher magnification of the rectangular box in J1)

chromosomal DNA in the adherently cultured and suspension-cultured in MPC-coated dish showed a similar pattern during culture (Fig. 6–1 and –2). It should be noted here that, similar to the respiratory activity, the MEP stimulated incorporation of ^3H -thymidine (Fig. 6–3). Cell proliferation during each day amounted to the increase of respiratory activity during the culture (Fig 5A).

Isolation and characterization of the 6 M-4-TR7 cell line, a derivative of 6 M-4

Because the 6 M-4 cells appeared to be morphologically heterogeneous, we attempted to isolate a

homogeneous cell population by clonal selection using the dilution method and the soft agar culture methods. As shown in Fig. 7A, most parental MDCK cells were not able to proliferate in soft agar plates (marked by filled triangle). However, at least two cell populations were found in 6 M-4 cells. Namely, one that was unable to proliferate but survived in soft agar (Fig. 7B, arrow), and another that had the ability to grow in soft agar (Fig. 7C, asterisk). Because of the ability of cells to grow in soft agar has been an indicator of the malignancy potential (Lu et al. 2004), the former cell line that showed an inability to proliferate in soft agar was focused and designated as

Fig. 3 Size distribution of 6 M-4 and parental MDCK cells in suspension culture. The confluent monolayer cultures were dispersed with trypsin, re-suspended in the MEM-10 and transferred to MPC-coated flasks. Then the cells were microscopically observed and the maximal diameter was measured after 3 (A) and 7 (B) days of culture. Each size of the aggregates was plotted in order of

size, from small (left) to large (right). Marks are for 6 M-4-TR7 (closed circle) and parental MDCK (open circle). Most of the parental MDCK cells were dead on the 3rd (A) and the 7th (B) day of the culture. Many larger aggregates were observed in the 6 M-4 populations at day 3 (A) and at day 7 (B)

Fig. 4 MEP-resistant 6 M-4 cells are anoikis-resistant 6 M-4 and parental MDCK cells cultured adherently were detached from the dishes with trypsin and transferred to the non-adherent MPC-coated dishes (A and B) or to normal adherent polystyrene dishes (C and D). After culturing for 8 h, the cells were collected as described in Materials and methods, and subjected to binding reaction for FITC-Annexin V followed by FACS analysis. For control experiments, adherently grown 6 M-4 and MDCK

cells were directly stained with FITC-Annexin V immediately after detachment by trypsin, and subjected to FACS analysis (E and F). G and H show FACS analysis of the cells without staining with Annexin V. All these cells were stained with propidium iodide (PI) as well, so that cells exhibiting positive signals for both of Annexin V (FL1) and PI (FL2) are those under apoptosis with inverted cell membranes. The Percentage of the total cells in quadruplet is indicated in the figures (A, B, C and D)

Fig. 5 Respiratory activities of 6 M-4 cells grown in the presence of MEP. Adherently grown 6 M-4 (A) and parental MDCK (B) cells were detached by trypsin and aliquots (10^4 cells) were transferred to a 96-well polystyrene plate in the absence (Adhesion) or presence of MEP (Suspension + MEP), as indicated in the figure. After culturing for the indicated time (in days, X-axis), redox

activities were assayed using alamarBlueTM (fluorescence intensity of reduced alamarBlue, Y-axis in both A and B) as described in Materials and methods. In suspension condition 6 M-4 cells (A) but not parental MDCK (B) cells increased redox activity during culture in contrast to adhesion condition where 6 M-4 cells and parental MDCK cells showed the same activity

Fig. 6 DNA synthesis activities of 6 M-4 cells grown under adherent and non-adherent conditions. 6 M-4 cells were cultured in adherent (polystyrene) dishes (left), in non-adherent (MPC-coated) dishes (middle), or in polystyrene dishes in the presence of MEP (right). The cells were prepared and cultured as shown in Figure 5 but incubated with ³H-thymidine for 18 hrs every day. Incorporation of ³H-thymidine into high molecular weight

DNA was measured as described in Materials and methods. Greater incorporation of ³H-thymidine into the suspension cells in the presence of MEP (right). Cell proliferation during each day amounted to the increase of respiratory activity during the culture (Fig 5A). Regardless the culture condition is used, ³H-thymidine incorporation per se decreased toward cell confluence

6 M-4-TR7. Subconfluent 6 M-4-TR7 cells grow with a spindle-shaped branching morphology, which is slightly different from parental MDCK. However, these cells adopted an epithelial-like cuboidal and squamous morphology upon contact inhibition. Epithelial cells express the cytokeratin intermediate filament and E-cadherin, a component of adhesion and tight junction. On the other

hand, elongated or stellate-shaped mesenchymal cells are characterized by the expression of vimentin intermediate filament. To see epithelial traits in the 6 M-4-TR7 cells, we examined the expression of the above proteins and actin. As shown in Fig. 8, 6 M-4-TR7 cells express actin (A), E-cadherin (C), cytokeratin (E) and vimentin (G) as do the parental MDCK cells (B,D,F and

Fig. 7 Selection of cells survived as a single cell in the soft agar plate. MDCK cells (A) and 6 M-4 cells (B and C) were cultured in soft agar plates for 10 days. Most MDCK cells died and lost their glossy appearances (A, marked by filled triangles). Three types were seen in 6 M-4 cells; dead

cells (B, filled triangles), alive as glossy single cells (B, arrows), and proliferating cells (C, asterisks). Cells that survived as a single cell (marked by arrows in B) were recovered and cultured adherently (termed 6 M-4-TR7 cells, see text)

Fig. 8 Expression of parental MDCK traits in 6 M-4-TR7 cells. 6 M-4-TR7 and parental MDCK cells grown adherently on the LAB-TEK II Chamber Slide were fixed with methanol and stained with FITC-labeled phalloidin (A and B), anti-E-cadherin (C and D), anti-cytokeratin (E and F) and anti-vimentin (G and H) antibodies. Except for phalloidin, FITC-labeled second antibodies were used for the detection of the proteins. Red PI (1 $\mu\text{g}/\text{ml}$) accumulated in the nucleus. Expression of molecules examined showed essentially the same traits in both cells

H). Thus, the results indicate that the 6 M-4-TR7 cells sustained the epithelial phenotype of parental MDCK cells.

Susceptibilities to tumor necrosis factor (TNF) and verotoxin (VT)

Because parental MDCK cells are resistant to both of TNF- α and verotoxin VT1, we examined the susceptibilities of the MDCK derivative cells to those factors. As assayed by the respiratory activity, the sensitivity to TNF- α was characteristic in 6 M-4-TR7, while parental MDCK and the Vero cells, used as a control, were not affected (Fig. 9A). Respiratory activities of parental MDCK cells were not inhibited by TNF- α , but rather stimulated. The binding of Annexin V (Fig. 9B) and staining nuclei with PI (Fig. 9C) followed by flow cytometric analysis revealed that TNF- α induces typical apoptotic patterns in 6 M-4-TR7 cells.

VTs are a family of subunit protein toxins derived from *Escherichia coli*, which are associated with hemolytic uremic syndrome. Subunit B of VT recognizes the cell surface glycosphingolipid, Gb3, to mediate the internalization of the A subunit, which inhibits cellular protein synthesis. Figure 10A shows the sensitivities of MDCK and its derivative cells to the treatment with VT1, as assayed by respiratory activities. While MDCK cells did not respond to VT1, 6 M-4-TR7 cells showed sensitivities to it. Vero cells, a positive control, showed the highest sensitivity. Next, the expression of Gb3 was examined using FITC-labeled anti-Gb3 (CD77) antibodies (Fig. 10B). As it is apparent from the figure, parental MDCK cells do not bind anti-Gb3 antibody, whereas the 6 M-4-TR7 cells bind to the antibody. The binding of the antibodies to 6 M-4 cells was less effective as compared to 6 M-4-TR7 cells. This might be the result of heterogeneous populations of 6 M-4 cells. The expression of Gb3 in derivative cells

Fig. 9 Susceptibility of 6 M-4-TR7 to TNF- α . Susceptibilities to recombinant TNF- α were assayed by redox activity (A), binding of Annexin V (B) and flow cytometric analysis of apoptotic cell appearance (C). A: Ten thousand of the cells were seeded in 96-well plates in the presence of the indicated concentrations of rmTNF- α . On day 4, dead cells in the medium were removed by aspiration and the resulting adherent cells were assayed for redox activities by incubating with alamarBlue for 4 h. Values were expressed as the percent of the control (without TNF- α). Marks are for 6 M-4TR7 (diamonds), parental MDCK

(filled circles), and Vero cells (open circles). B: Binding of Annexin V to 6 M-4-TR7 (1) and MDCK (2) cells adherently grown for 15 h in the presence (10 ng/ml) of TNF- α . Cells were stained with FITC-Annexin V and PI, as described in the Materials and methods. C: FACS analysis of 6 M-4-TR7 (1) and MDCK (2) cells stained with PI after ethanol fixation. The filled histograms represent control cells. The solid lines indicate TNF- α treated cells as above. The typical apoptotic fraction was detected in 6 M-4-TR7 cells

Fig. 10 Susceptibility, of 6 M-4-TR7 to VT1. A: Redox activities of 6 M-4, MDCK and Vero cells grown in 96-well plates in the presence of VT1. Symbols and growth conditions were the same as those in the legend to Fig. 9, B: Expression of Gb3 (CD77) in the 6 M-4-TR7. Cells were cultured on the LAB-TEK II Chamber Slides as described in the legend to Fig. 8, but fixed with 2% paraformaldehyde and stained with anti-Gb3 antibody followed by FITC-labeled second antibody and PI. 6 M-4-

TR7 cells were Gb3 positive in contrast to the parental MDCK cells being negative. C: Thin layer chromatogram of cellular glycolipids. The total glycolipids extracted from 5×10^6 cells and separated by TLC as described in the Materials and methods. M: the standard mixture of Gb1, Gb2, Gb3, Gb4 and Gb5. The bands of Gb3 were detected in the lanes of Vero (positive control) and 6 M-4-TR7 but not in the parental MDCK lane

was confirmed by the presence of 2-bands corresponding to neutral lipids in the TLC chromatography, as shown in Fig. 10C.

Production of influenza virus in MDCK and 6 M-4-TR7 cells

To see if the suspension culture of 6 M-4-TR7 cells was suitable for production of influenza viruses, different types of influenza virus, A/H3N2, A/H1N1, B and a clinical isolate A/H3/undefined N, were inoculated. Table 1 shows a summary of the virus production assayed with hemagglutination (HA titer) and infectivity (TCID₅₀). The influenza viruses examined were efficiently amplified in non-adherently cultured 6 M-4 and 6 M-4-TR7 cells. 6 M-4-TR7 cells produced higher titers in the presence of MEP than trypsin.

Test for the absence of mycoplasma

Absence of mycoplasma with the parental MDCK, 6 M-4, and 6 M-4TR7 was confirmed with nested PCR (data not shown) described in the “Materials and methods” section.

Discussion

In the present study, we isolated novel MDCK-derived cell line, 6 M-4-TR7, which is able to proliferate adherently and non-adherently. The cells were selected as those that survived after long-term culturing in the presence of bacterial MEP. The successful use of bacterial proteinase has also been reported for disperse from *Bacillus polymyxa* (Nagata et al. 1986). The authors described the wide variation among cell lines in the mode of the proteinase action, e.g., detachment of cells from the matrix, dispersion of cells into single cells or into clusters and the cytotoxicity. They also reported that epithelial cell lines of kidney origin, MDCK and RK13, were relatively resistant to disperse treatment. Disperse detached MDCK cells as a sheet in the absence of serum. However, the MEP used in this study was able to detach MDCK cells to single cells under the same culture conditions. In the presence of serum, MEP detached the adherent MDCK cells as a sheet, similar to the action of disperse. Thus, the effects on MDCK cells differ between disperse and MEP.

Table 1 Evaluation of Influenza virus production

Exp 1	Time course of HA titer ^a					
	Suspension/MEP	day 1 1	day 3 64	day 4 64	day 5 64	day 6 64
Exp 2	Comparison of HA titer among cells, culture conditions and Flu strains ^b					
			Flu strains			
			A/NC/H1N1	A/Pa/H3N2	A/Mori/H3N ^c	B/St
	6M-4-TR7	Suspension/MEP	64	256	256	1024
		Suspension/Trypsin	16	32	32	64
		Adhesion/Trypsin	16	16	2	32
	MDCK	Adhesion/Trypsin	4	8	2	16
		$\frac{6M-4-TR7 \text{ suspension/MEP}^d}{MDCK \text{ adhesion/trypsin}}$	16	32	128	64
Exp 3	Correlation of HA and infectious titer (TCID ₅₀) ^e					
			HA		TCID ₅₀	
	Suspension/MEP		128		10 ^{6.3}	
	Suspension/Trypsin		2		10 ^{4.5}	

^a Exp 1 shows the time course of virus antigen productions. An aliquot of suspension culture of 6 M-4 inoculated virus stock at day 0 was removed along the culture period. Virus antigen appeared after days 3

^b Exp 2 shows comparison of the productivity of virus in 6 M-4-TR7 and parental MDCK cells in three different conditions: suspension culture in MEM-10 containing MEP (25 µg/ml), suspension culture in MPC flask with the standard concentration of trypsin (2 U/ml) and adherent culture with the trypsin (2 U/ml). The influenza virus strains used were as follows: NC: A/New Caledonia/20/1999/H1N1, Pa: A/Panama/2007/1999/H3N2, Mori: A/Morioka/O-68N/2000/H3 (Morioka City, Japan). B/Shandong/7/1997. HA titers of the virus in non-adherent TR7 supplemented with the MEP were higher than that in adherent state of TR7 and MDCK cells with trypsin

^c N type was not defined

^d Exp 3 proved the infectivity of virus produced in suspension culture. The virus was recovered on day 3 and the TCID₅₀ was evaluated using the procedure described in the Materials and methods section

^e The median tissue culture infectious dose (TCID₅₀/100/µl) of the virus suspension was evaluated with MDCK cells cultured in rolling test tubes. Development of a cytopathic effect (CPE) was observed for 10 days

MDCK cell line is known to be comprised of diverse cells with different properties, for example, with different growth signaling pathways (Taub et al. 1979; Husted et al. 1986; Wunsch et al. 1995; Arthur 2000). That a morphologically different cell line or population (6 M-4) can be isolated from MDCK cells after long-term exposure to MEP might support this fact. Whether the anoikis-resistant cells isolated in this study resulted from the selection of pre-existing cells in MDCK population or from the accumulation of genetic mutations that caused an alteration of survival signaling under MEP stress is of great interest. DNA-micro array analysis showed that the synthesis of interleukin 8 (IL-8) mRNA (data not shown) was constitutively increased in 6 M-4-TR7, which gave us a key to elucidate how the MEP effects on MDCK, e.g. regarding the signaling pathway of the MEP versus 6 M-4-TR7.

The isolated cell line 6 M-4-TR7 showed sensitivities to TNF- α and Vero toxin VT1.

Originally, MDCK cells were resistant to both of TNF- α and VT1. Recombinant TNF- α induced cytotoxicity in canine kidney tumor cells, but it did on MDCK cells only when they were treated with actinomycin D (Aoki et al. 1997). Verotoxin, also called Shiga toxin (Stx), binds to the specific cell surface receptor globotriaosylceramide (Gal α 1-4Gal β 1-4glucosylceramide, Gb3). Increased sensitivities to VT have been observed in many multidrug-resistant ovarian carcinoma and astrocytoma cells that are correlated with the increased synthesis of Gb3 (Lala et al. 2000). Wild type MDCK is resistant to VT because it synthesizes Gb5 instead of Gb3, and it becomes sensitive only after treatment with short-chain fatty acids or with tumor promoter 12-O-tetradecanoylphorbol 13-acetate (Sandvig et al. 1991). The 6 M-4-TR7 cells acquired susceptibility to VT and the expression of Gb3. These facts may imply that the 6 M-4-TR7 cells show common phenotypes to some malignant cells. However, we

think it may be possible that the 6 M-4-TR7 cells are still in a pre-malignant state, because the cells express cytokeratin, vimentin and E-cadherin, as does the parental MDCK, and because those cells cannot grow in soft agar. The ability of cells to grow in soft agar has been an indicator of the malignancy potential (Lu et al. 2004).

The cultivation system of the influenza virus suitable for the production of vaccines or diagnostic antigens has been restricted to the use of MDCK, PER.C6 (Pau et al. 2001), and chicken embryonic stem (Taylor et al. 1988) cells in culture and embryonated hen eggs. The 6 M-4-TR7 cells described here, which can be cultured non-adherently in suspension without any solid or porous carriers, have many advantages for the large-scale cultivation of the influenza virus. As shown in Table 1 (Exp 2), the MEP appears to have promoting effect for the growth (as shown in HA titers) of influenza viruses compared to that with trypsin. Low concentration of MEP, which is not made by parental MDCK cells in non-adherent state, had no effect on influenza virus propagation (data not shown). In general, HA titers of the virus in non-adherent 6 M-4-TR7 were relatively higher than that in adherent state even with trypsin. Thus, we think that non-adherent condition of 6 M-4-TR7 plus the *S. griseus* MEP makes cellular condition up regulated for viral growth, which has to be further studied.

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