WAVE2- and microtubule-dependent formation of long protrusions and invasion of cancer cells cultured on three-dimensional extracellular matrices

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Invadopodia, small protrusions formed at ventral membranes of several types of invasive cancer cells upon contact with the extracellular matrix (ECM), are implicated in cell invasion; however, the relationship between invadopodia formation and cell invasion through the ECM is still unknown. To correlate the formation of membrane protrusions and cell invasion, a three-dimensional (3-D) gel culture system with native collagen type-I matrix overlaid with a thin basement membrane equivalent (Matrigel) was made. Human breast cancer cell line MDA-MB-231 formed long protrusions in addition to small protrusions reminiscent of invadopodia and migrated into the collagen layer. Comparative analyses with other cancer cell lines indicate that cellular ability to form long protrusions, but not small protrusions or invadopodia, correlates with cellular invasiveness in the 3-D culture. Some of the long protrusions in MDA-MB-231 cells appeared to extend from the adherence membrane, implying that they are derived from small protrusions. The formation of long protrusions and invasion, as well as the formation of invadopodia, required WAVE2 in MDA-MB-231 cells. Accumulation of tubulin was observed in long protrusions but not in invadopodia. Correspondingly, a microtubule-stabilizing agent, paclitaxel, suppressed the formation of long protrusions and invasion, but not the formation of invadopodia, in MDA-MB-231 cells. These results suggest that long protrusions formed in a WAVE2- and microtubule-dependent manner may identify the cells at the later stage of invasion, possibly after the formation of invadopodia in the 3-D cultures. (*Cancer Sci* 2008; 99: 2252–2259)

Invasive cancer cells possess abilities to break down the surrounding extracellular matrix (ECM) and to migrate through it, which are elemental processes in cancer metastasis.[1] Since these processes take place in non-cancerous cells engaged in inflammatory responses or wound healing, identification of invasive behaviors specific to cancer cells is crucial to elucidate the mechanism of cancer invasion and to prevent cancer metastasis. One of the invasive responses of cancer cells upon contact with the ECM is the formation of specialized actin-cytoskeletal protrusions towards the ECM, termed invadopodia ([2–6] for reviews). Invadopodia were initially described with v-*src*-transformed fibroblasts,[7] and formed in several invasive cancer cells cultured on the ECM. Whereas the cell shapes of cancer cells invading the three-dimensional ECM are quite diverse,[8,9] the morphology of invadopodia is similar among the cells. They extend from the ventral cell membranes to the ECM and are 0.1–0.8 μm in diameter and over 2 μm in length.[10,11] Although a specific molecular marker that is exclusively present in invadopodia has not been identified so far, invadopodia are characterized by focalized degradation of the surrounding ECM and colocalization of multiple proteins that are involved in cell adhesion, signal transduction especially downstream of src kinase, regulation of the actin cytoskeleton and cellular motility.[2,5,6] One of the key components for rearrangement of the actin cytoskeleton in invadopodia is neural Wiskott–Aldrich Syndrome protein (N-WASP).[12,13] The N-WASP and related WASP family Verprolin-homologous protein (WAVE) family proteins that activate the Arp2/3 complex are important regulators of the initiation of actin polymerization ([14] for a review). In two-dimensional cell cultures, N-WASP and WAVE family proteins have been shown to work in the formation of certain types of actin-based protrusions in the cell periphery, such as filopodia and lamellipodia.[15,16] Invadopodia are formed through the N-WASP–Arp2/3 signaling pathway.[12,17] Corresponding to these results, activation of N-WASP, as assessed by fluorescence resonance energy transfer analysis to detect the conformational change of N-WASP, was demonstrated to occur at the site of invadopodia formation.[18] Despite accumulating knowledge about the mechanism and dynamics of invadopodia formation,[11,13] it is as yet unknown whether the formation of invadopodia represents cell invasiveness through the ECM.[3,4] This may be partly because the formation of invadopodia has been studied in most cases with thin layers of ECM, the thickness of which is not sufficient to observe cell invasion into the ECM.

To identify the protrusive structures that closely link to invasion through the ECM, we made a three-dimensional (3-D) culture system consisting of a thick layer of native collagen type-I gel overlaid with a thin layer of basement membrane equivalent (Matrigel) that mimics the configuration of epithelial tissues. When cultured on Matrigel, an invasive breast cancer cell line, MDA-MB-231, which was previously reported to form invadopodia on gelatin films,[11,19–23] invaded the collagen type-I layer. In addition to small protrusions reminiscent of invadopodia that penetrated Matrigel, MDA-MB-231 cells extended longer protrusions into collagen type-I over several tens of micrometers. Comparative analysis of the behaviors of other cancer cell lines revealed that the cellular ability to form long protrusions rather than the small protrusions or invadopodia correlates with cell invasiveness in our 3-D culture system.

Materials and Methods

Cells and cell cultures. Human breast cancer cell line MDA-MB-231 was obtained from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK). Human fibrosarcoma cell line HT-1080 was provided by the Health Science Research Resources Bank (Osaka, Japan). MDA-MB-231 cells and human breast cancer cell line MCF-7 were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal

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Abbreviations: N-WASP, neural Wiskott–Aldrich Syndrome protein; WAVE, WASP family Verprolin-homologous protein; MMP, matrix metalloproteinase; Arp 2/3, actin-related proteins 2 and 3.

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Table 1. Small interference RNA (siRNA) and antibodies used in RNA interference experiments in the present study

Target	siRNA	Antibody for detection of protein
N-WASP	GAAAUGUGUGACUAUGUCUTT TTCUUUACACACUGAUACAGA	Cell signaling, rabbit MAb (30D10)
WAVE1	UCCUUCGUAUUUCUUUGAUTT TTAGGAAGCAUAAAGAAACUA	Santa Cruz, goat pAb (L-19)
WAVE2-1†	AAACCAGAUCCUCUUUGGUUGUCCA UUUGGUCUAGGAGAAACCAACAGGU	Chemicon, rabbit pAb (AB4226)
WAVE3	CUUCUACAUCAGAGCAAAUTT TTGAAGAUGUAGUCUCGUUUA	Santa Cruz, goat pAb (N-16)
WAVE2-2†	UAUCAUUGGAGGCGGAGGUGGCGGA AUAGUAACCUCCGCCUCCACCGCCU	Chemicon, rabbit pAb (AB4226)
WAVE2-3†	AUCAGGGUGAGGUGGGAAAGAUGGG UAGUCCCACUCCACCCUUUCUACCC	Chemicon, rabbit pAb (AB4226)
Control	GACGUGAAACCGAAGAACGTT TTCUGCAGUUUGGCUUCUUGC	

†Invitrogen stealth siRNA.

bovine serum (FBS). HT-1080 cells and human squamous carcinoma cell line A431 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. GM6001, its negative control compound and paclitaxel were obtained from Calbiochem (San Diego, CA, USA).

Cell cultures on Matrigel-collagen type-I double-layered 3-D gel. To form 3-D matrix gels, 100 μL of 2 mg/mL of neutralized collagen type-I gel (Nitta Gelatin, Osaka, Japan) containing final 1 × RPMI 1640 medium, 1% bovine serum albumin (BSA) and 20 ng/mL of epidermal growth factor (EGF) was spread on a 2 cm × 2 cm well on a glass slide (AR Brown, Tokyo, Japan), hardened and then coated with 15 μL of Matrigel (Becton-Dickinson, Frankline Lakes, NJ, USA) diluted with an equal volume of RPMI 1640 medium using a glass rod. For visualization of the Matrigel layer, 15% (v/v) of Matrigel conjugated with Alexa Fluor 568 using a labeling kit (Molecular Probes, Eugene, OR, USA) was mixed with Matrigel. The gels were placed in a CO_2 incubator at 37°C for 1 h, and then overlaid with 500 μL of RPMI 1640 medium containing 0.05% bovine serum albumin (BSA), 10 μg/mL transferrin and 20 ng/mL EGF for equilibration.

For 3-D culture, the cells were washed once with phosphate buffered saline (PBS) and cultured for 16 h to 24 h in medium containing 0.05% BSA and 10 μg/mL transferrin. The cells were detached from plastic dishes using PBS containing 0.2% (w/v) ethylenediaminetetraacetic acid (EDTA) and resuspended in RPMI 1640 medium containing 0.05% BSA and 10 μg/mL of transferrin. Two to 3×10^4 cells in 500 μL of the medium were plated onto the 2 cm × 2 cm 3-D gel.

Invadopodia formation. Coverslips were coated with gelatin conjugated with Oregon Green 488 (Molecular Probes) as described previously.[11] The cells were plated on the coverslips with RPMI 1640 medium containing 10% FBS and incubated for 18 h.

Cell immunofluorescence and analyzes of protrusion formation and cell invasion. The cells were fixed with 4% paraformaldehyde in PBS for 1 h and soaked in Tris-buffered saline (TBS) for at least 1 h. They were then permeabilized with 0.05% saponin in TBS and used for staining with fluoroscein isothiocyanate (FITC)- or Rhodamine-conjugated phalloidin (Molecular Probes), anticortactin rabbit polyclonal antibody (Santa Cruz Biotechnology, H-191; Santa Cruz, CA, USA) or antiβ-tubulin monoclonal antibody (Chemicon MAB3408; Temecula, CA USA). In most cases, staining was performed in TBS containing 3% skim milk and 0.1% Tween-20 at 4°C for 16 h. After washing, cells were stained with appropriate combinations of FITC- or Rhodamine-labeled secondary antibodies (Molecular Probes). The stained cells were examined using a laser scanning confocal microscope (Carl Zeiss, model LSM 5 Pascal; Jena, Germany).

To determine the percentage of cells with protrusions or invaded cells, 8–12 fields of the culture were randomly selected and scanned using a 20× objective lens with resolutions of 512 × 512 pixels per plane. The thickness of slices, and therefore vertical resolution, was 2 μm. 3-D images of the cells in a field were examined for the formation of protrusions or invasion using the criteria described in the Results section. At least 300 cells were examined in each culture and, unless otherwise noted, the average and standard deviation of the results were calculated based on at least triplicate independent experiments.

For the analyses of invadopodia, the stained cells were scanned using a 63× oil-immersion objective lens. The cells with at least one overlapping site of focally degraded-gelatin and a punctate aggregate of F-actin were judged to form invadopodia

RNA interference. The small interference RNA (siRNA) used in this study are as shown in Table 1. Solution containing 12.5 μL of 20 μM siRNA solution was mixed with 10 μL of LipofectamineRNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) in 1 mL of serum-free RPMI 1640 medium in 6-cm diameter dishes and allowed to form a complex for 15 min at room temperature. Then 4 mL of cell suspension in RPMI 1640 containing 10% FBS was mixed with the siRNA complex, resulting in a final concentration of 50 nM siRNA. The cells were transfected for 24 h and then the medium was replaced with RPMI 1640 medium containing 0.05% BSA and 10 μg/mL of transferrin. After 24 h, the cells were detached by PBS containing 0.05% EDTA and plated onto 3-D gels or lyzed in a buffer containing 0.2% NP-40 to extract proteins, as described below.

Western blot analyses. The cells were lyzed in ice-cold lysis buffer (20 mM HEPES–KOH pH 7.2, 142.5 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2% NP40, protease inhibitor cocktail [Roche, Mannheim, Germany]), incubated on ice for 10 min, and then homogenized by gently passing through a 23-gauge syringe 10 times. The lysates were centrifuged at 15 000 × g for 10 min and the supernatant was recovered. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories; Hercules, CA, USA). Typically, 10 μg of protein was resolved by electrophoresis in a sodium dodecylsulfate (SDS)–10% polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated with the first antibodies overnight at 4°C. The antibodies used for the detection of target proteins are shown in Table 1, and they were detected using an enhanced chemiluminescence system (GE Healthcare; Buckinghamshire, UK). For the detection of WAVE3, the membrane was treated with 0.5% glutaraldehyde before detection by the secondary antibody.

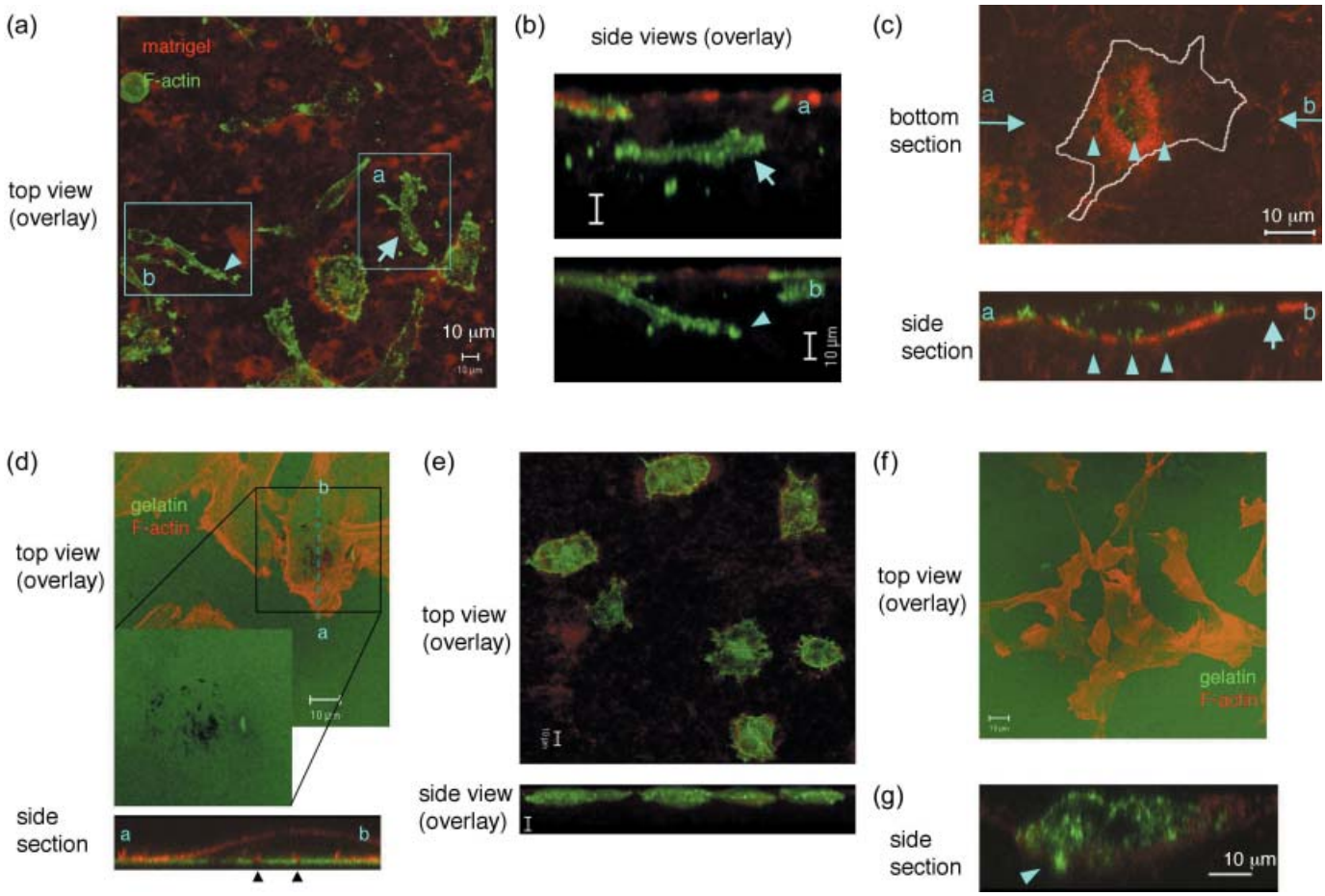

Fig. 1. Formation of protrusions and invasion into the extracellular matrix (ECM) of MDA-MB-231 cells and MCF-7 cells. (a) Top view of the MDA-MB-231 cells cultured on Matrigel (Alexa Fluor 568 labeled; red) spread on native collagen type-I matrix. Cells were cultured for 18 h and fixed, and then stained with fluoroscein isothiocyanate (FITC)–phalloidin (green) for F-actin. The image was reconstructed by overlaying confocal planner images. (b) Side views of invaded (upper panel, the cell in the region-a of panel a) or protrusion forming- (lower panel, the cell in the region-b of panel a) MDA-MB-231 cells shown in (a). (c) Small protrusions in MDA-MB-231 cells cultured on 3-D gel. In the upper panel, a section at the cell bottom is shown with a trace of the upper cell edge. In the lower panel, small protrusions at ventral membranes of the cells are shown. The side section of the cells was reconstructed along the dashed line (a–b) in the upper panel. Arrowheads mark the protrusions that penetrated the Matrigel layer. An arrow indicates a gap in the Matrigel outside of the cells. (d) Invadopodia formation of MDA-MB-231 cells (stained with Rhodamine-phalloidin, red) into Oregon Green 488-conjugated gelatin (green). In the inset of top view, focalized degradations of gelatin are shown. In the lower panel, the side section of invadopodia along the dashed line (a–b) in the top view is shown. Arrowheads mark invadopodia. (e) MCF-7 cells cultured on the 3-D gel. Reconstructed top and side views are shown in the upper and lower panels, respectively. (f) Representative image of MCF-7 cell (stained with Rhodamine-phalloidin, red) cultured on Oregon Green 488-conjugated gelatin film (green). (g) Small protrusions in MCF-7 cultured on 3-D gel.

Results

Formation of protrusions and invasion in MDA-MB-231 cells cultured on Matrigel-coated collagen type I gel. To assess the formation of protrusions and invasion in a 3-D culture system, human breast cancer line MDA-MB-231 plated on Alexa Fluor 568 labeled-Matrigel was fixed and stained with FITC–phalloidin for the actin cytoskeleton after incubation for 18 h (Fig. 1a). Some cells completely invaded into the collagen gel (Fig. 1b, upper panel). The cells attached to Matrigel carried two types of protrusions; long protrusions with the length of several 10 μm within the collagen gel (Fig. 1b, lower panel) and small protrusions (several micrometers in length and 1–2 μm in diameter) penetrating through the Matrigel into collagen from their ventral surface (Fig. 1c, arrowheads).

Small protrusions at adherence membranes associated with punctate sites lacking Matrigel, reminiscent of invadopodia, formed into a gelatin film (Fig. 1d). However, Matrigel did not uniformly spread on the collagen, forming patches of dense and sparse regions (Fig. 1a). Further, it was contracted by the cells,[24] and eventually torn around the cells (Fig. 1c, an arrow). These make it difficult to determine whether the small protrusions are invadopodia, because it is possible that the small protrusions are formed into the gaps of Matrigel in the absence of matrix-degrading activity. To test this, we examined with human breast cancer MCF-7 cells. They did not invade into the collagen type-I matrix (Fig. 1e) nor formed invadopodia (Fig. 1f). Nevertheless, the small protrusions at ventral membranes were also formed in MCF-7 cells (Fig. 1g, an arrowhead).

We then analyzed the long protrusions in detail. Here, to define 'long' protrusion consisting of the actin cytoskeleton, we employed the following criteria: (1) the cell body is in contact with Matrigel but a protrusion tip is located in collagen type-I matrix, separate from Matrigel; and (2) the protrusion is longer than the diameter (for round cells) or thickness (for flat cells; approximately 5–10 μm) of the cell body. To identify whether cells would constantly form long protrusions and invade thereafter, we further cultured MDA-MB-231 cells for 48 h. Prolonged incubation resulted in an increase in the number of invaded cells and concomitant decrease in the number of cells with long protrusions (Fig. 2a).

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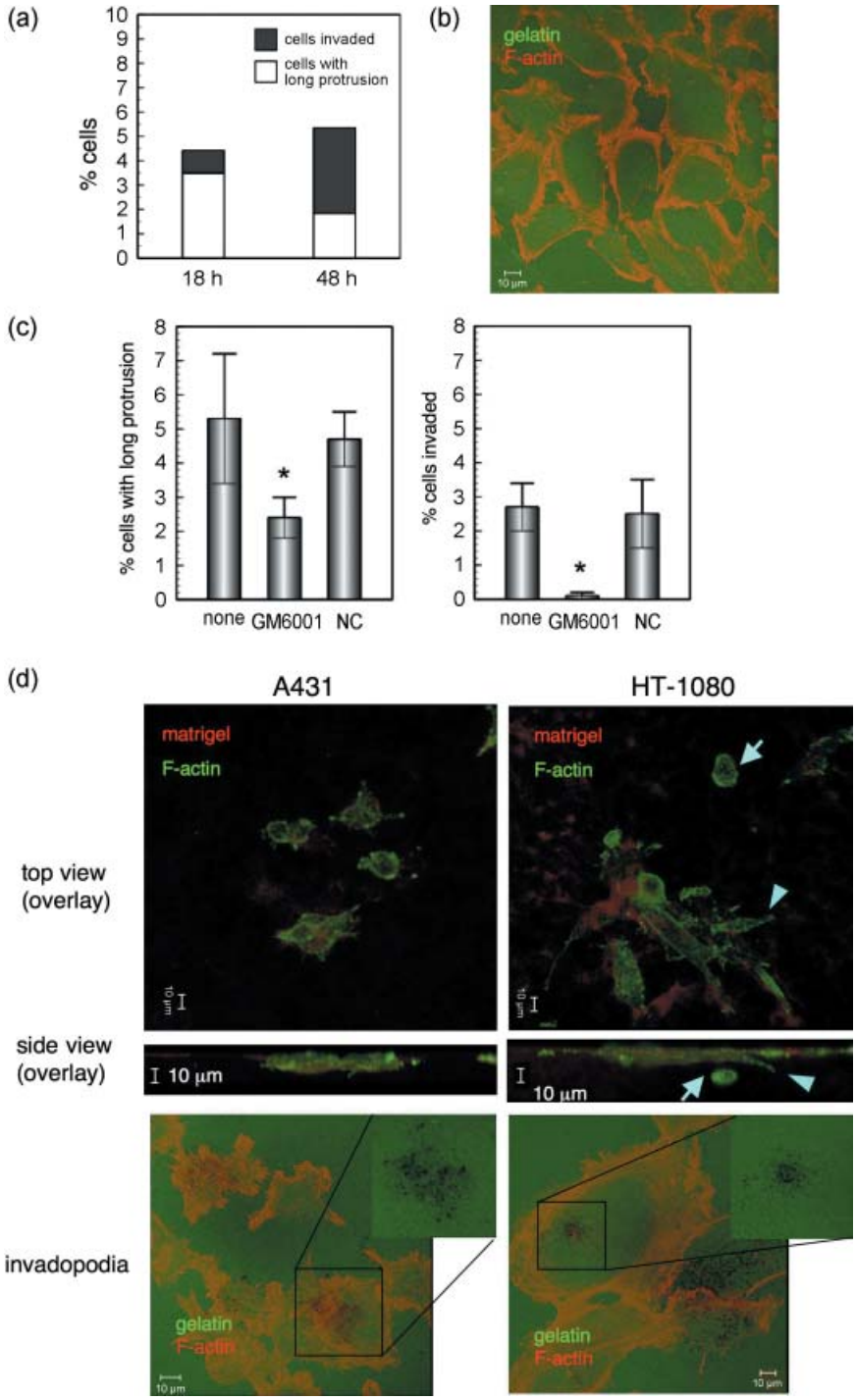

Fig. 2. (a) Time changes of cells with long protrusions and those invading the collagen type-I layer counted at 18 h and 48 h after plating. The result is an average of two independent cultures. (b) Effect of matrix metalloproteinase (MMP) inhibitor GM6001 (10 mM) on the formation of invadopodia in MDA-MB-231 cells. (c) Effect of MMP inhibitor GM6001 (10 mM) on the formation of long protrusions and invasion of MDA-MB-231 cells. NC denotes treatment of the cells with an inactive GM6001 derivative (10 mM) as the negative control. Asterisks mark the results with *P*-values of Student's *t*-test under 0.05. (d) Formation of protrusions and invasion on 3-D gel or invadopodia formation in A431 human squamous carcinoma cells (left panels) or HT-1080 human fibrosarcoma cells (right panels). The cultures were made and analyzed as described in the legend of Figure 1. Top panels and middle panels show top and side views of the culture, respectively. Bottom panels show invadopodia. In the panel of HT-1080 cells, arrowheads indicate cells carrying a long protrusion and arrows mark the invaded cell. Scale bar represents 10 μm.

Correlation of the formation of long protrusions with invasiveness of cancer cells cultured on 3-D gel. To determine whether the formation of long protrusions represents cellular invasiveness with activity to degrade the ECM or the amoeboid mode of cells without it,[8,25,26] we examined the effect of GM6001, a potent inhibitor of matrix metalloproteinase (MMP),[27,28] on the formation of invadopodia or long protrusions in MDA-MB-231 cells. GM6001 at 10 μM completely suppressed formation of invadopodia (Fig. 2b) as well as invasion (Fig. 2c). It significantly reduced the formation of long protrusions, but by 50% (Fig. 2c). These results suggest that some of the long protrusions are formed in an MMP-independent manner, if not the cells are in the amoeboid mode.

We next investigated the behavior of other cancer cell lines after incubation on 3-D gel for 18 h. A human squamous carcinoma cell line A431 did not form long protrusions or migrate into the collagen layer (Fig. 2d), with up to 72 h of cultivation (data not shown). They could form invadopodia into gelatin (Fig. 2d). By contrast, a human fibrosarcoma cell line HT-1080 carrying strong activity to form invadopodia (Fig. 2d), formed long protrusions similar to those formed in MDA-MB-231 cells and invaded into the collagen layer (Fig. 2d). These two cell lines on 3-D gel both formed small protrusions at their adherence membranes (data not shown).

Dependence of formation of long protrusions and invasion on WAVE2 in MDA-MB-231 cells. We then asked from which of the

protrusions, small protrusions at adherence membranes or protrusions at cell periphery such as lamellipodia, the long protrusions are derived. We examined the morphology of MDA-MB-231 cells at 5 h after plating onto Matrigel. To identify extending protrusions, we examined for colocalization of F-actin and cortactin, which indicates active actin polymerization.[29] Of several 10 cells examined, approximately half of the cells appeared to extend long protrusions containing discrete regions with colocalized F-actin and cortactin from a central part of ventral membrane (Fig. 3a), implying that they might be extended from small protrusions. The other half of the cells appeared to extend long protrusions from junction of cell periphery and adherence membrane (Fig. 3b), raising a possibility that they are derived from peripheral protrusions.

To further obtain an insight into the origin(s) of long protrusions, we examined which of the N-WASP/WAVE family proteins is involved in the formation of long protrusions in MDA-MB-231 cells. A previous report indicates that invadopodia formation depends on N-WASP, but not WAVE family proteins that participate to lamellipodia formation.[17] Reduction of N-WASP expression by RNA interference (Fig. 4a) exhibited no significant

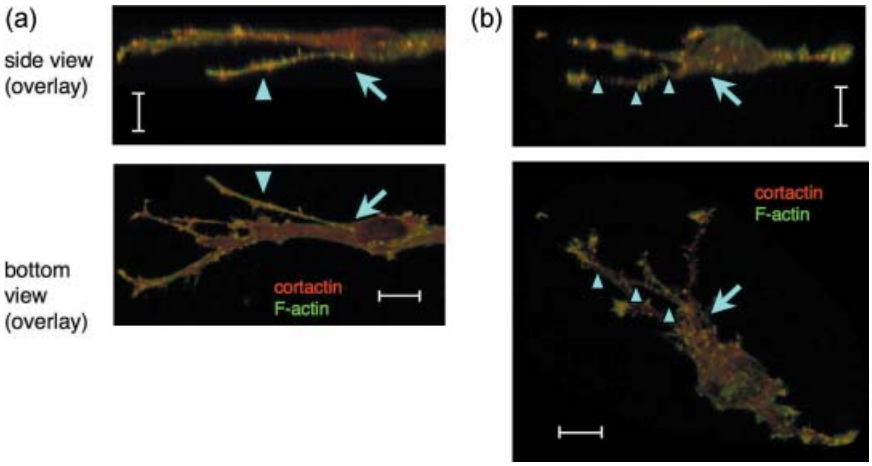

Fig. 3. Morphology of MDA-MB-231 cells at 5 h of cultivation of 3-D gel. The cells were stained with fluoroscein isothiocyanate (FITC)–phalloidin (green) and anticortactin polyclonal antibody (red). (a) A long protrusion (arrowheads) judged to be extended from a central part of adherence membrane (arrows). (b) A long protrusion (arrowheads) judged to be extended from junction of cell periphery and adherence membrane (arrows). Scale bars represent 10 μm.

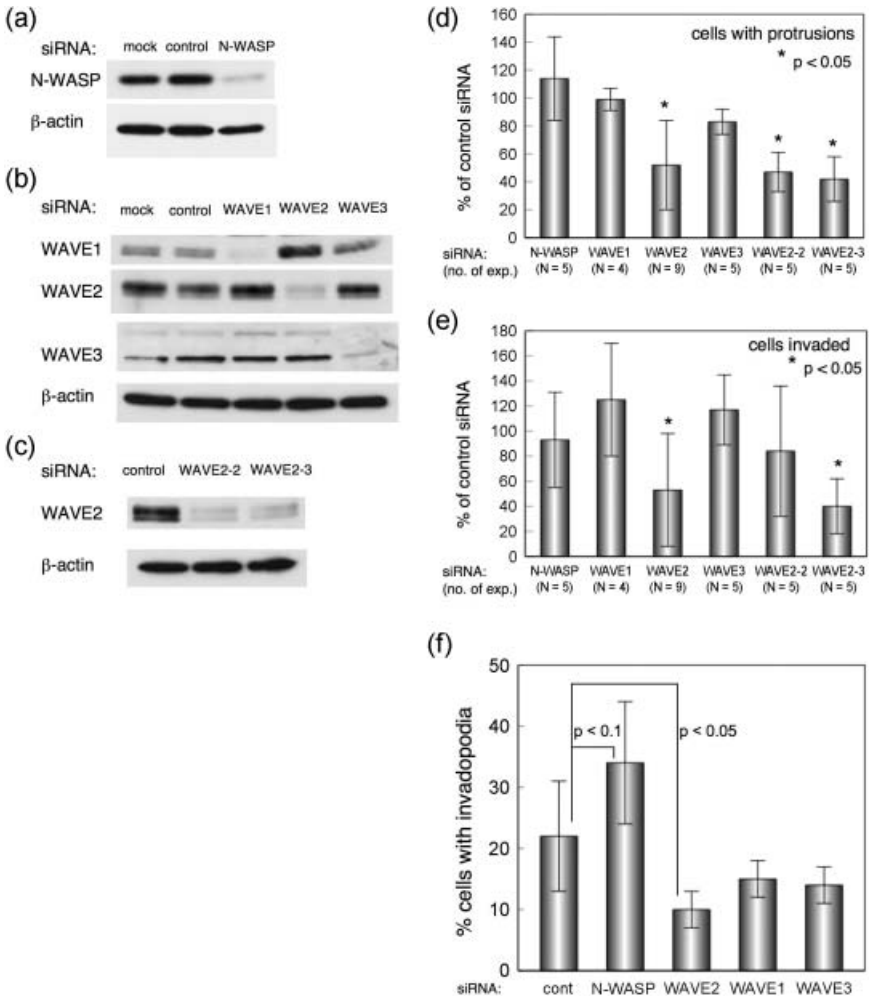

Fig. 4. (a–c) Reduction of neural Wiskott–Aldrich Syndrome protein (N-WASP) or WASP family Verprolin-homologous protein (WAVE) family proteins by RNA interference in MDA-MB-231 cells. Cells were transfected with small interference RNA (siRNA) for 24 h and further cultured for 24 h, then part of the cells was used to extraction of proteins and the rest were plated on 3-D gel. Western blot analyzes of N-WASP (a), WAVE family proteins (b) and WAVE2 (c) expression. (d and e) Effect of siRNA on the formation of long protrusions and invasion. Cells transfected with siRNA were cultured on 3-D gel for 18 h. Results of cells transfected with specific siRNA are normalized to those of cells treated with control siRNA as 100 in each experiment. Averages of the results of repeated experiments (N: number of experiments) are shown with standard deviations (error bars). Asterisks mark the results with *P*-values of Student's *t*-test less than 0.05. (f) Effect of siRNA on the formation of invadopodia. Cells transfected with siRNA for 48 h were plated onto Oregon Green 488 conjugated-gelatin film and cultured for 18 h. The cells with one or more sites in which focally degraded-gelatin and a punctate aggregate of F-actin were judged to form invadopodia. Averages of the results of four independent experiments are shown with standard deviations (error bars). *P*-values of Student's *t*-test for the difference between the results with control siRNA and either N-WASP or WAVE2 siRNA are noted in the figure. Those with control siRNA and siRNA for WAVE1 or WAVE3 exceeded 0.1.

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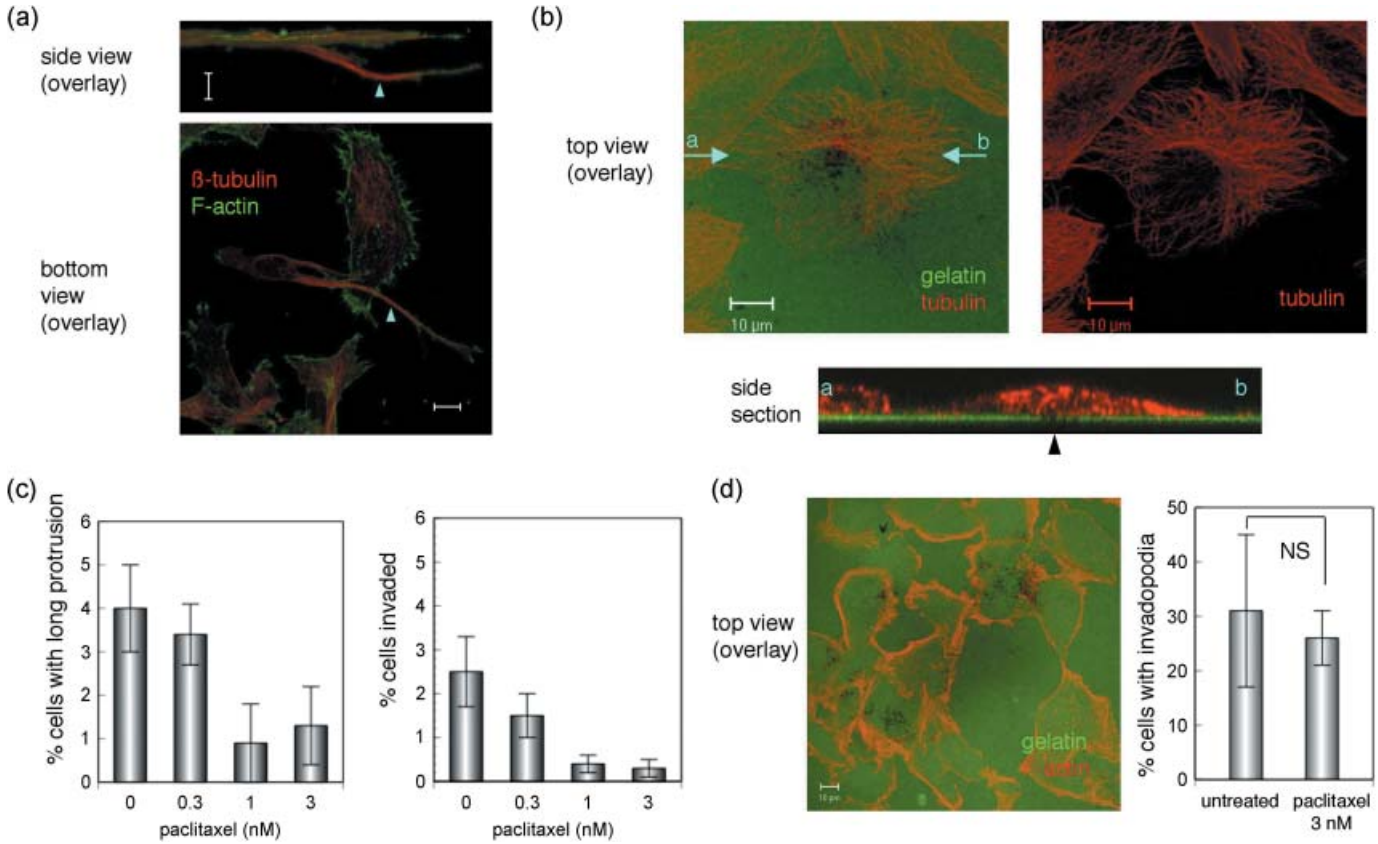

Fig. 5. (a) Localization of tubulin (red) in MDA-MB-231 cells (stained by fluoroscein isothiocyanate (FITC)–phalloidin; green) cultured on 3-D gel. Arrowheads mark long protrusions. (b) Localization of tubulin (red) in MDA-MB-231 cells cultured on Oregon Green 488 conjugated-gelatin film (green). In the right panel, tubulin alone is shown. In the bottom panel, the side section of the cells was reconstructed along the dashed line (a–b) in the upper panel. Arrowheads mark focally degraded gelatin. (c) Effect of paclitaxel on the formation of long protrusions or invasion of MDA-MB-231 cells cultured on 3-D gel. Cells were cultured with or without 3 nM paclitaxel for 18 h (d). Effect of paclitaxel on the formation of invadopodia in MDA-MB-231 cells. In the left, top view of the culture is shown. The cells (stained by Rhodamine–phalloidin; red) were cultured for 18 h on Oregon Green 488 conjugated-gelatin (green) in the presence of 3 nM paclitaxel. In the right, averages of the results of three independent experiments are shown with standard deviations (error bars). NS, not statistically significant.

effect on the formation of long protrusions (Fig. 4d) as well as the invasion of MDA-MB-231 cells (Fig. 4e). By contrast, the reduction of WAVE2 expression (Fig. 4b) decreased the number of cells with long protrusions and invading cells by approximately 50%, respectively (Fig. 4d,e). In contrast, reduction of either WAVE1 or WAVE3 expression (Fig. 4b) had no significant effect on the invasive behavior of MDA-MB-231 cells (Fig. 4d,e). To confirm the effect of WAVE2 knockdown, we further examined using two other siRNA against WAVE2 (Fig. 4c). Reduction of WAVE2 by these siRNA suppressed the formation of long protrusions and invasion (Fig. 4d,e), indicating the necessity of WAVE2 in the formation of long protrusions and invasion in MDA-MB-231 cells. This implies that the formation of invadopodia in MDA-MB-231 cells themselves depends on WAVE2, rather than N-WASP. To know whether this is the case, we examined the effect of reduction of N-WASP/WAVE family proteins. Reduction of WAVE2 but not other family members resulted in a significant decrease of the number of cells with invadopodia (Fig. 4f).

Accumulation of tubulin to long protrusions and microtubule-dependent formation of long protrusions. The result of A431 cells suggested that the formation of long protrusions and invasion into 3-D gels requires machineries in addition to those for invadopodia formation. To identify them, we explored which proteins are concentrated in long protrusions. WAVE2 that participates in the formation of long protrusions appeared not to be specifically accumulated in the long protrusions (data not shown). A previous report showed tubulin accumulation in the protrusions formed in human lung carcinoma LCLC103H cells cultured on a collagen 3-D matrix.[30] Immunostaining with antitubulin antibody revealed the accumulation of tubulin in long protrusions (Fig. 5a), but not in invadopodia (Fig. 5b). To test the necessity of microtubules for the formation of long protrusions and/or invasion of MDA-MB-231 cells, cells were incubated with paclitaxel, a microtubule-stabilizing agent.[31] Paclitaxel at 3 nM effectively inhibited the formation of long protrusions (Fig. 5c, left panel) as well as invasion into collagen type-I matrix (Fig. 5c, right panel), without loss of cell viability (data not shown). By contrast, the formation of invadopodia was not affected by paclitaxel (Fig. 5d), suggesting that microtubules are additionally required for the formation of long protrusions.

Discussion

In the present report, we explored the membrane protrusions formed in cancer cells linked to cell invasion through the ECM. We constructed a 3-D gel consisting of thick native collagen type-I matrix overlaid with a thin Matrigel layer. MDA-MB-231 cells plated on 3-D gel migrated into the collagen. We identified two types of protrusions into the ECM, small (several micrometers in length and 1–2 μm in diameter) protrusions at the ventral

Table 2. Summary of the formation of protrusions and invasion of cancer cell lines in the present study

Cell line	3-D cultures		Gelatin film	
	Invasion	Small protrusions	Long protrusions	Invadopodia†
MDA-MB-231	+ (2.4%)	+	+ (5.1%)	33%
MCF-7	–	+	–	none
A431	–	+	–	27%
HT-1080	+ (nd)	+	+ (nd)	70%

†Number of cells examined: MDA-MB-231 666; MCF-7, 299; A431, 443; HT-1080, 188; nd, not determined quantitatively.

membrane and long (longer than the cell body and with several 10 micrometers in length) protrusions. The small protrusions are morphologically reminiscent of invadopodia. But the small protrusions were also formed in MCF-7 cells that neither formed invadopodia nor invaded into the ECM. Gaps in the Matrigel, probably formed upon contraction of the matrix by the cells, may trap the small protrusions, resulting in the formation of sites that look like invadopodia. Therefore, the formation of small protrusions itself does not reflect cell invasiveness in our 3-D culture system. By contrast, the formation of long protrusions in the collagen type-I matrix correlated with cell invasiveness in 3-D gel. Using the criteria described in the Results section, of about 12 600 MDA-MB-231 cells that had been plated onto 3-D gel, 5.1% and 2.4% of the cells formed long protrusions and invaded the collagen, respectively, after cultivation for 18 h. Prolonged cultivation resulted in a decreased number of cells with long protrusions and increased number of invaded cells. This implies that cells form long protrusions upon contact with the gel and then invade it.

Comparative analyses with other cancer cell lines indicated that the cellular ability to form long protrusions rather than invadopodia reflects cell invasiveness in the 3-D culture system (Table 2). Highly invasive HT1080 cells that formed long protrusions migrated into the collagen layer; however, low-invasive MCF-7 cells and A431 cells that are reportedly invasive,[9] and form invadopodia (Fig. 2b) did not invade the collagen type-I matrix or form long protrusions. The inability of A431 cells to invade in this study is currently unknown. Only highly invasive cells may form long protrusions and invade the collagen layer in our 3-D culture system. The morphology of long protrusions is similar to that of highly metastatic cancer cells in the amoeboid mode during migration through the 3-D collagen matrix.[27] MMP inhibitor GM6001 almost completely suppressed invasion and invadopodia formation of MDA-MB-231 cells, whereas it reduced the formation of long protrusions only by 50%. The GM6001-insensitive moiety of long protrusions could be formed in the amoeboid mode, though participation of proteases other than MMP is yet to be evaluated.

The morphology of MDA-MB-231 cells after 5 h on 3-D gel implies that either small protrusions formed at ventral membrane or protrusions formed at cell periphery, such as lamellipodia extending into long protrusions. Considering the cellular ability to form invadopodia, some of the small protrusions in MDA-MB-231 cells could be invadopodia. In addition, both the formation of long protrusions and invadopodia formation were suppressed by the reduction of WAVE2 expression and the reduction of N-WASP expression did not inhibit the formation of invadopodia or long protrusions in MDA-MB-231 cells. These results strongly suggest that some of the long protrusions are derived from invadopodia formed in a WAVE2-dependent manner. This contradicts the previous report indicating that the formation of invadopodia requires N-WASP but not WAVE2.[12,17] The reason for this discrepancy remains currently unknown. It is possible that a member of N-WASP/WAVE family proteins involved in invadopodia formation is different among cell lines. It also could be due to the difference of the ECM components used for the detection of invadopodia.

Although the roles of WAVE2 in cell migration through lamellipodia formation have been extensively analyzed,[16,32,33] its participation in invasion or metastasis of cancer cells is yet to be established. WAVE2 has been previously shown to be necessary for cell invasion and metastasis of mouse melanoma cells.[34] In hepatocellular carcinoma, increased expression of WAVE2 is reported to correlate with vein invasion and poor prognosis.[35] In addition, colocalization of WAVE2 and Arp 2/3 complex is a risk factor for liver metastasis of colorectal carcinoma.[36] Our results indicating the suppression of invasion of MDA-MB-231 cells upon reduction of WAVE2 expression may further implicate the participation of WAVE2 in cancer cell invasion.

We found that a microtubule component, tubulin, was accumulated in long protrusions but not invadopodia. Paclitaxel, which stabilizes microtubules, effectively blocked the formation of long protrusions as well as invasion into collagen. Of the two possible precursors of the long protrusions, formation of lamellipodia has been previously shown to be regulated by microtubules.[31,37] By contrast, the formation of invadopodia was not affected by paclitaxel, suggesting that the organization of microtubules is necessary for the extension of long protrusions from invadopodia. The mechanism(s) that coordinates the formation of actin-based protrusions and microtubules is unknown at present. It has been supposed that microtubules deliver a wide variety of molecules, including membrane components, actin-organizing proteins and MMP, to the cell front, facilitating cell migration and invasion.[30,38–40] Also, it is proposed that microtubules liberate Rac guanosine triphosphatases upon polymerization to induce actin polymerization,[31] (reviewed in [41]). This proposal may coincide with our observation that WAVE2, which mediates Rac-dependent actin polymerization,[16,32,34,42] participates to the formation of long protrusions. Currently, the participation of Rac in the formation of long protrusions by MDA-MB-231 cells in 3-D cultures is under investigation.

In conclusion, we found that cellular ability to form long protrusions into the ECM correlates well with the cell invasiveness in the 3-D culture system employed here. Some of the long protrusions formed in an MMP- and WAVE2- dependent manner could be derived from invadopodia and may specify the cells at the later stage of invasion. The requirement of microtubules for the formation of long protrusions suggests that they are extended by the mechanism distinct from, or at least additional to, one for the formation of invadopodia. Further elucidation of the mechanism by which long protrusions are formed in the 3-D culture system will provide clues to the target molecules in the prevention of cancer cell invasion.

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