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Report

Adenovirus Fiber can Distribute Itself to the Cell Surface without Membrane Damage

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Fiber is an adenovirus (Ad) capsid protein that binds to coxsackievirus and Ad receptor. It is secreted by Ad-infected cells in the early infection stage, and it increases the permeability of the epithelial cells. Accordingly, fiber may facilitate the apical escape of Ad particles from the basolateral side in Ad-infected cells. However, its behavior in the Ad-infected cells remains unclear. Therefore, we investigated the behavior of fiber in the Ad-infected cells by fluorescence microscope analysis. Results showed that a higher proportion of fiber molecules were present in the apical side compared with that in the basolateral side, and electrical resistance, which represents cell–cell adhesion, remained unaffected in the Ad-infected cells. Furthermore, the association between fiber secretion and membrane damage was analyzed using annexin V and propidium iodide staining. We observed that fiber was distributed to the membrane surface without membrane damage. In addition, fiber distribution occurred in Ad-infected cells as well as in fiber-expressing cells. Therefore, fiber can distribute itself to the cell surface, and it plays a novel role in Ad infection. Further investigation of fiber distribution would be useful to completely elucidate Ad infection mechanism and develop antiviral strategies for Ad.

Key words adenovirus, fiber, cell lysis

INTRODUCTION

The first step in the Ad type 5 (Ad5) infection is the binding of the Ad capsid protein — fiber — to coxsackievirus and Ad receptor (CAR) in the host cell.[1)] Upon the binding of fiber to CAR, penton base (Pb) interacts with cellular integrins, thereby promoting viral internalization.[2)]

Numerous fiber and Pb molecules are secreted to the extracellular domain from Ad-infected cells in the early infection stage,[3)] and this capsid protein secretion may regulate Ad infection by receptor masking, which promotes Ad and host cell coexistence.[4)] In addition, fiber assists the escape of the virus to the environment by disrupting the epithelial barriers.[5)]

The mechanism of fiber and Pb secretion has been investigated in recent years. A recent study indicated that fiber secretion is regulated by the cytoskeletal network.[3)] Moreover, Pb is secreted from Ad-infected cells by a Golgi-independent mechanism,[6)] suggesting that the Ad capsid protein may be secreted via a specific mechanism.

Although Ad capsid protein secretion may play key roles in Ad life cycle, the behavior and correlation of Ad capsid protein secretion with cell lysis remains unclear.

In the present report, we have thoroughly investigated the behavior and secretion of the fiber protein in Ad-infected and fiber-expressing cells and results have suggested that we observed that this protein can distribute itself to the cell surface without membrane damage.

MATERIALS AND METHODS

Cell Culture Caco-2, Hela, 293, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells expressing green fluorescent protein (GFP) were generated using lentiviral vectors. Ad5 fiber-expressing 293 cells previously generated by us, were used.[7)] Ad5 fiber-expressing 293T cells were generated by transfecting with pCMV-Ad5fiber by the calcium phosphate method.[8)]

Virus Wild type Ad5 (Wt-Ad5) was obtained from ATCC VR-5 (USA). Ad5 vectors contain a cytomegalovirus promoter–driven firefly luciferase expression cassette (Ad5 v). The virus particle titers of Ad vectors were determined as per the method described by Maizel et. al.[9)]

Transepithelial Electric Resistance (TER) Analysis Caco-2 monolayer cells were cultured in Transwell® Permeable Supports (0.4 μm polycarbonate membrane, 6.5 mm insert, 24-well plate, Corning). TER values were monitored using Millicell® ERS-2 and calculated as follows: TER = Total

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resistant (Ω) × the effective area (cm²) − Blank resistant (Ω) × the effective area (cm²)

Cell ELISA Caco-2 monolayer cells and Ad-infected Caco-2 cells were cultured in the apical side of the transwell at indicated time. Cells were fixed in 4% paraformaldehyde and were further blocked with 1% BSA in PBS. Anti-CAR mouse antibody (1:8000; clone RmcB, Merck) or anti-fiber mouse antibody (1:2000; clone 4D2, Neo Marks) was used to treat the upper (apical side) or lower (basal side) compartment in transwell. After washing with PBS, cells were incubated with horseradish peroxidase–labeled goat anti-mouse IgG in the upper or lower compartment of the transwell. Further, the cells were washed with PBS and incubated with 3,3′,5,5′-tetramethylbenzidine in the upper or lower compartment of the transwell. The enzyme reaction was stopped by 1 N H_2SO_4. The absorbance was determined at 450 nm using a microplate reader (Varioskan Flash; Thermo Fisher Scientific).

Immunofluorescence Microscopy Caco-2 cells were seeded into a 24-well plate. After 7 d, they were treated with Wt-Ad (300 virions/cell) for 24 h. Further, the cells were washed with culture medium and incubated for 2 d. After 2 d, the cells were washed with PBS. Cells were fixed in 1% formaldehyde and incubated in 0.1% Triton X-100. Fixed cells were blocked with 5% BSA in PBS and incubated with an anti-fiber mouse antibody (1:1500; clone 4D2, Neo Marks). After washing with PBS, cells were incubated with the goat anti-mouse IgG (H+L) conjugated with Alexa fluor 680. Images of cells were generated using a confocal laser scanning microscope system (ECLIPS TI-E, Nikon). The apical and basal sides were distinguished from each other based on three-dimensional stack images taken using a confocal laser scanning microscope (data not shown).

Flow Cytometry Membrane damage and fiber intensity in Ad5v- or Wt-Ad-infected cells were assessed at indicated time post infection. For detecting early apoptosis and late apoptosis in Ad-infected cells, cells were treated with annexin V (Invitrogen) or PI (Invitrogen) and were washed with PBS. For staining the fiber, cells were incubated with anti-fiber mouse antibody (1:400; clone 4D2, Neo Marks). Subsequently, cells were washed with PBS and incubated with allophycocyanin- or PE-labeled secondary antibody. The stained cells were washed thoroughly and analyzed by FACS Calibur (Becton Dickinson) and Cell Quest software (Becton Dickinson).

RESULTS

Fiber is Secreted to the Apical Side in Caco-2 Monolayer without Cell–Cell Junction Disruption CAR was mainly localized on the basal side in Caco-2 cells (Fig. 1A). Meanwhile, following Wt-Ad5 infection, a higher proportion of fiber molecules were present in the apical side compared with that in the basal side in Ad-infected Caco-2 cells (Fig. 1B, C). Fiber reportedly decreases TER in Ad-infected cells by disrupting the tight junction.[5] To assess the integrity of cell–cell

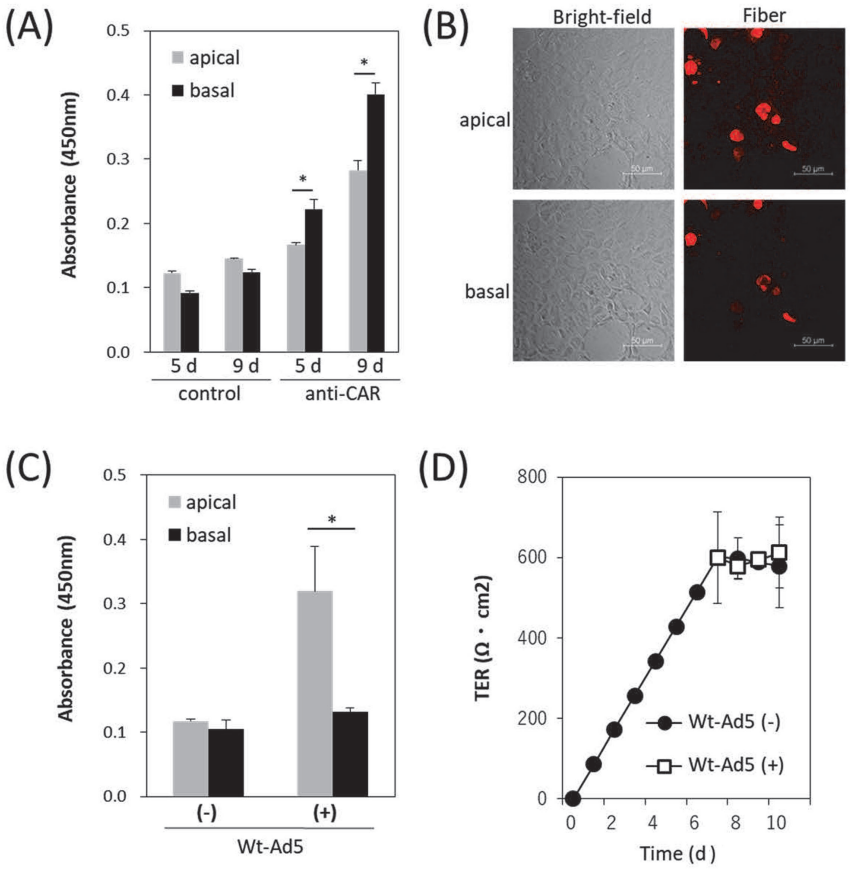

Fig. 1. Fiber Localization and Effect on Tight Junction Permeability in Ad-Infected Epithelial Cells

Coxsackievirus and Ad receptor intensity in Caco-2 monolayer cells was measured by cell ELISA after 5 or 9 d of incubation (A). Caco-2 cells were infected using Wt-Ad5 at 300 virions/cell after 7 d of incubation. Fiber localization levels on the apical or basal cell surface were detected using immunostaining (B) and cell ELISA analysis (C). On day 7, Caco-2 monolayer cells were infected with Wt-Ad5 at 300 virions/cell. Transepithelial electrical resistance (TER) was monitored at indicated time (D). Fig. 1A, C, and D represent the mean ± SD; n = 3. *, $P < 0.05$.

junctions in Wt-Ad5-infected cells, we measured TER in Ad-infected Caco-2 cells. Interestingly, TER was not affected in fiber-expressing cells (Fig. 1D). Therefore, fiber was secreted to the apical side rather than the basal side in Caco-2 monolayer cells.

Fiber is Secreted to the Cell Surface without Membrane Damage Fiber was secreted to the apical surface without cell–cell junction disruption (Fig.1). To investigate the association between fiber secretion and membrane damage, we stained Ad vector-infected cells using annexin V and propidium iodide (PI). Fiber molecules were not detected on the cell surface at 10 hours post infection (hpi) after the infection (Fig. 2A, B; Table 1). Although abundant fiber molecules were detected on the cell surface at 24 hpi, annexin V- or PI-positive cells were not detected in Ad vector-infected 293 cells (Fig. 2A, B; Table 1). Similar findings were observed in Wt-Ad-infected HeLa cells (Fig. 3A, B). Thereafter, we examined the relationship between fiber secretion and Ad infection dose. Although the proportion of fiber molecules on the cell surface increased depending on Ad infection dose whereas annexin V- and PI-positive cells were only detected at 1000 virions/cell (Fig. 2C). Therefore, these results suggest that fiber is secreted to the cell surface without cell lysis.

Fiber can Distribute Itself to the Cell Surface The abovementioned findings indicate that fiber may be secreted to the cell surface without cell lysis in Ad-infected cells; however, the factor that causes fiber secretion remains unknown. We focused on fiber molecules and examined fiber secretion using Ad5 fiber-expressing 293T cells. Fiber molecules were detected on the cell surface in Ad5 fiber-expressing cells (Fig. 4A). In addition, we verified whether that fiber was secreted to a culture supernatant in Ad5 fiber-expressing cells

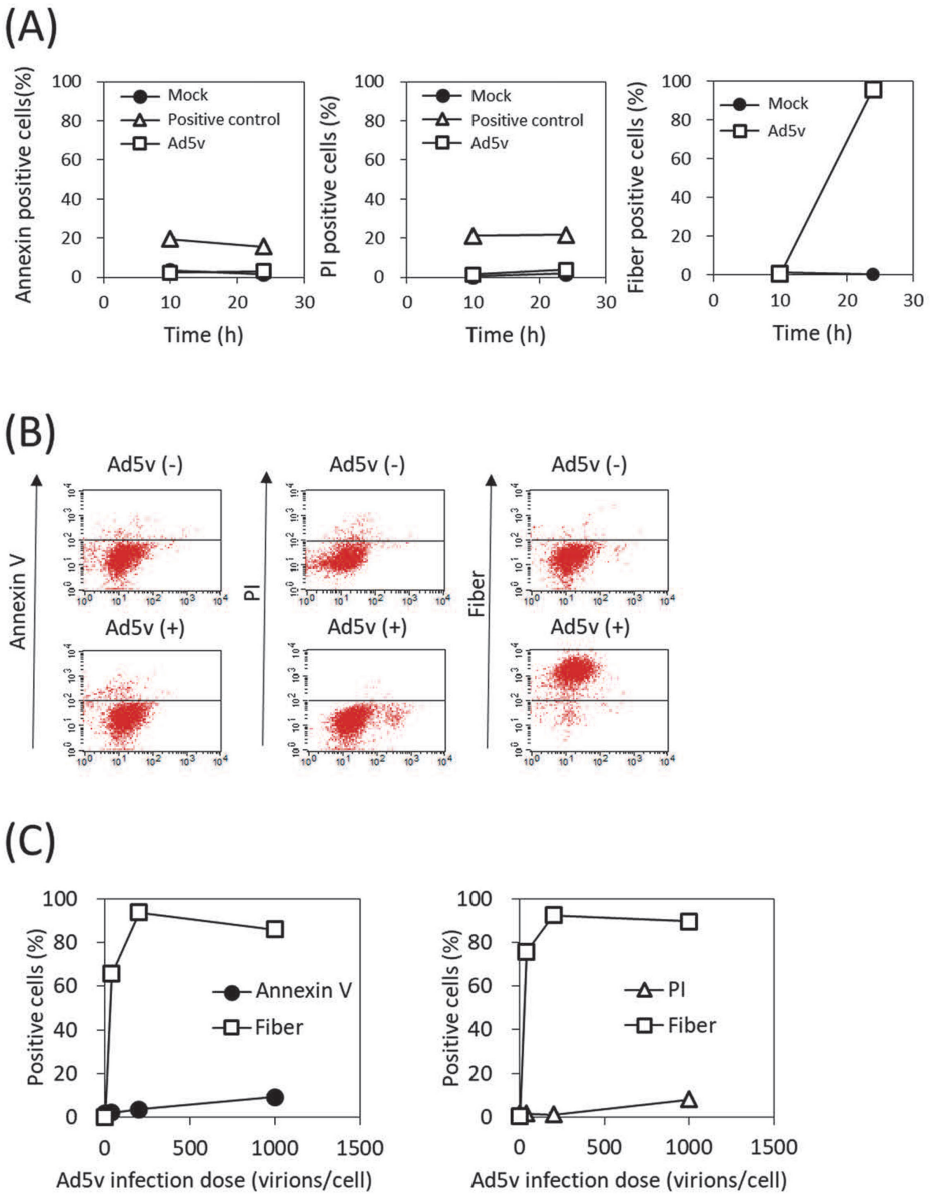

Fig. 2. Relationship Between Fiber Secretion and Cell Damage in Ad5-Infected Cells

Ad5v infected 293 cells at 200 virions/cell. After 10 or 24 h, the 293 cells were stained using annexin V, PI, or anti-fiber antibody and detected using FACS (A). The cells that were incubated with 0.1% FBS in DMEM were used as the positive control of cell lysis. Analysis data are shown in Table 1. Fluorescence intensity of stained cells at 24 hpi was analyzed using FACS (B). Ad5v-infected 293 cells at 40, 200, and 1000 virions/cell. After 24 h, 293 cells were stained by annexin V, PI, or anti-fiber antibody and detected using FACS (C). All data are presented as the mean ± SD of three experiments.

Table1. Analysis of (%) positive cells in Fig.2 A

		10 h	24 h
Early apoptosis (Annexin V)	Mock	3.26 ± 0.07	1.67 ± 0.15
	Positive control	19.62 ± 0.55	15.66 ± 0..43
	Ad infection	2.48 ± 0.12	3.20 ± 0.07
Late apoptosis (Propidium iodide)	Mock	0.46 ± 0.42	1.93 ± 0.27
	Positive control	21.27 ± 0.62	21.77 ± 0.44
	Ad infection	1.30 ± 0.29	3.87 ± 0.23
Fiber	Mock	1.15 ± 0.10	0.55 ± 0.05
	Positive control	8.41 ± 0.14	9.39 ± 0.71
	Ad infection	0.48 ± 0.03	95.78 ± 0.20

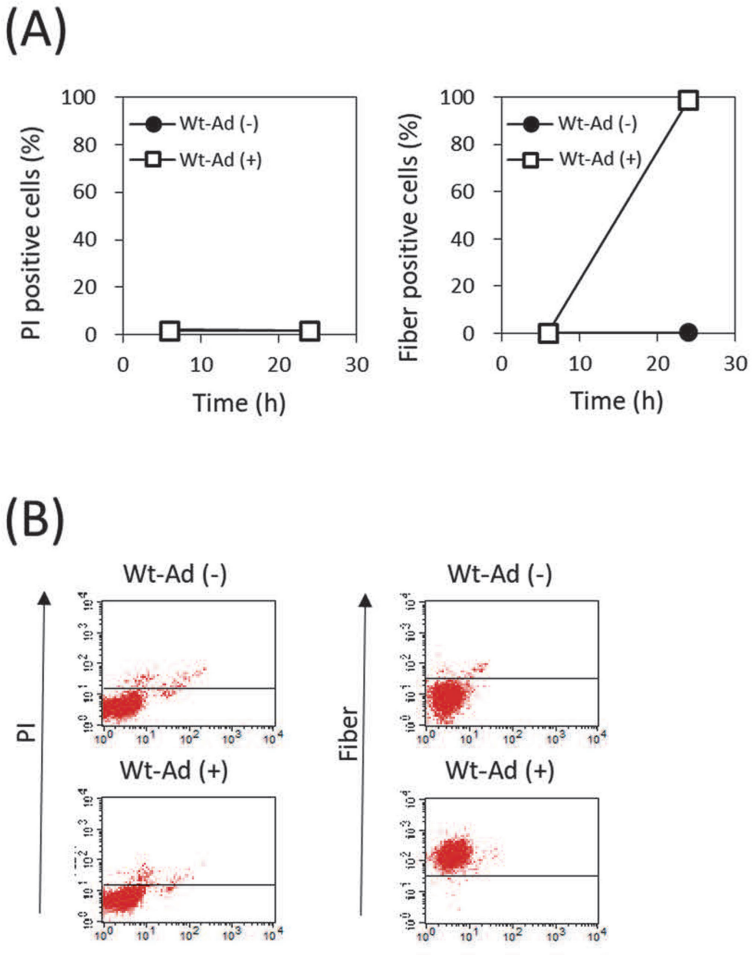

Fig. 3. Relationship Between Fiber Secretion and Cell Damage in Wt-Ad-Infected Cells

HeLa cells were infected with Wt-Ad at 200 virions/cell. After 6 or 24 h, HeLa cells were stained using PI or anti-fiber antibody and detected using FACS (A). Fluorescence intensity of stained cells at 24 hpi was analyzed using FACS (B). All data are presented as the mean ± SD of three experiments.

using the transwell assay. Ad5 fiber-expressing cells can share a medium with non-expressing cells, whereas these cultured cells are separated by the membrane of the transwell assay (Fig. 4B). Fiber was detected in Ad5 fiber-expressing cells, but co-cultured cells were not observed (Fig. 4C). These findings suggested that fiber did not get transferred to the other cells by being released in the culture supernatant. Thus, we suspect that fiber got transferred to the cell surface by itself.

DISCUSSION

As several reports have stated, Ad structural protein is released from Ad-infected cells. Fiber and Pb molecules spread from an infected cell to the neighboring cells because these proteins help disseminate the virus to more distant cells via receptor masking on neighboring cells.[6] CAR is localized in epithelial tight junction and plays a key role in the junctional barrier function.[10] The abundance of fiber molecules helps the virus escape to the apical side by binding to CAR and interfering with the cell–cell junction.[5] Therefore, releasing the Ad structural protein plays a key role in Ad secondary infection. However, the mechanism of releasing Ad structural protein remains unclear. Therefore, we focused on the behavior of fiber molecules. Fiber existed in the apical side and that it exerted no effect on the TER in Ad-infected epithelial cells (Fig. 1B, C, D). Walters RW *et al.* reported that

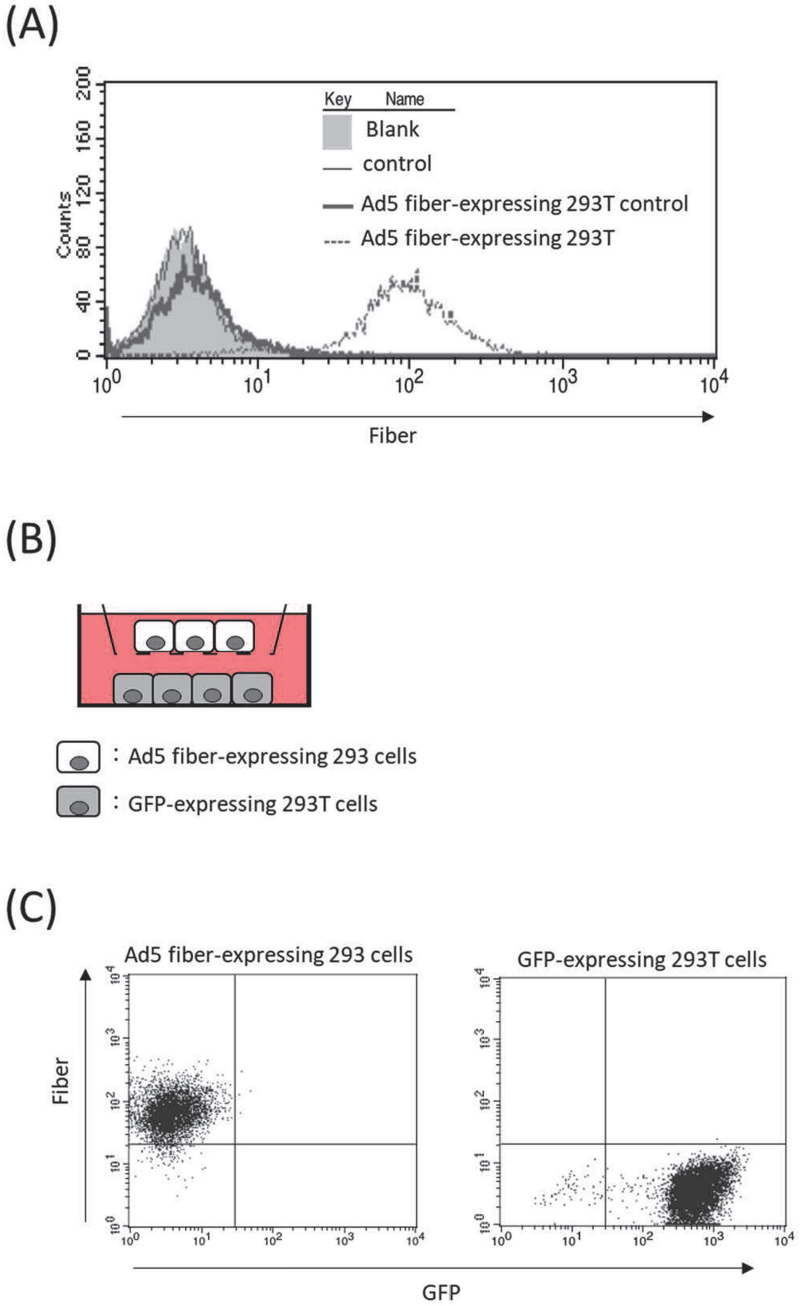

Fig. 4. Fiber Secretion in the Culture Supernatant from Fiber-Expressing Cells

After 48 h, Ad5 fiber-expressing 293T cells were stained using anti-fiber antibody and detected using FACS (A). Control samples were treated only by anti-mouse antibody-conjugated R-PE, whereas the blank samples were not treated with an antibody. Fiber-expressing 293 cells were co-cultured with GFP-expressing 293T cells in the transwell for 1 d (B). These cells were stained using anti-fiber antibody and detected using FACS (C). All data are presented as the mean ± SD of three experiments.

although TER was decreased by the basolateral knob protein, apical knob protein failed to alter TER,[5] conforming to our current results. In addition, the proportion of fiber molecules in the apical side was higher than that on the basal side. Meanwhile, the expression of CAR on the apical side was smaller than that on the basal side (Fig. 1A). Therefore, fiber secretion exerted no effect on TER in Ad-infected cells, suggesting that fiber is secreted to the apical side without cell lysis. Although Ad-infected cells secrete fiber to the cell surface in the early infection stage,[3] fiber is detected at 48 and 78 hpi in the supernatants of Ad5-infected cells.[4] In addition, cell membrane disruption and Ad particle release for secondary infection would require approximately 50 h.[11] We have elucidated similar data in our experiments (data not shown).

To clarify the association between membrane damage and fiber secretion to the apical side, we performed a serial assessment of fiber molecules on the cell surface and cell membrane damage by annexin V and PI staining. We identified that fiber was distributed to the cell surface without membrane damage on Ad vector- and Wt-Ad-infected cells at 24 hpi (Fig. 2, 3). Therefore, fiber may be distributed to the apical side in Ad-infected cells without cell lysis.

Furthermore, we investigated whether fiber molecules are present in the cell culture supernatants of Ad5 fiber-expressing cells. Our data showed that fiber molecules were not detected on the cell surface of co-cultured cells that shared a medi-

um with fiber-expressing cells, but fiber molecules were distributed to the cell surface in fiber-expressing cells (Fig. 4C). Yan Y *et al.* reported that fiber molecules are actively secreted and propelled along the cytoskeleton highway toward the plasma membrane in Ad-infected cells.[3] Our data suggested that fiber molecules can bind to the receptor as well as distribute itself to the cell surface without membrane damage. Thus, fiber molecules may have been distributed to the cell surface via an unknown mechanism.

Furthermore, the reason for fiber molecules being distributed on the cell surface without cell membrane damage remains unclear. Rebetz J *et al.* reported that fiber plays the role of Ad and host cell coexistence via receptor masking.[4] These reports and our data indicated that the distribution of fiber molecules without membrane damage assists in avoiding apoptotic cell clearance and may contribute to Ad and host cell coexistence via persistent fiber distribution on the cell surface. Overall, fiber can distribute itself to the cell surface in the apical side without cell lysis in Ad-infected cells. Therefore, the present study contributes toward elucidating the mechanism of Ad infection and Ad secondary infection.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

1) Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, **275**, 1320–1323 (1997).
2) Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins avb3 or avb5 promote adenovirus internalization but not virus attachment. *Cell*, **73**, 309–319 (1993).
3) Yan Y, Zhang B, Hou W, Lin H, Rebetz J, Hong SS, Wang Y, Ran L, Fan X. Active and separate secretion of fiber and penton base during the early phase of Ad2 or Ad5 infection. *Virology*, **505**, 172–180 (2017).
4) Rebetz J, Na M, Su C, Holmqvist B, Edqvist A, Nyberg C, Widegren B, Salford LG, Sjogren HO, Arnberg N, Qian Q, Fan X. Fiber mediated receptor masking in non-infected bystander cells restricts adenovirus cell killing effect but promotes adenovirus host co-existence. *PLoS One*, **4**, e8484 (2009).
5) Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, Welsh MJ. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell*, **110**, 789–799 (2002).
6) Trotman LC, Achermann DP, Keller S, Straub M, Greber UF. Nonclassical export of an adenovirus structural protein. *Traffic*, **4**, 390–402 (2003).
7) Koizumi N, Mizuguchi H, Sakurai F, Yamaguchi T, Watanabe Y, Hayakawa T. Reduction of natural adenovirus tropism to mouse liver by fiber-shaft exchange in combination with both CAR- and alphav integrin-binding ablation. *J. Virol.*, **77**, 13062–13072 (2003).
8) Chen C, Okayama H. High-Efficiency Transformation of Mammalian Cells by Plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745–2752 (1987).
9) Maizel JV Jr, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology*, **36**, 115–125 (1968).
10) Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc. Natl. Acad. Sci. USA*, **98**, 15191–15196 (2001).
11) Yakimovich A, Gumpert H, Burckhardt JC, Lütschg VA, Jurgeit A, Sbalzarini IF, Grebera UF. Cell-free transmission of human adenovirus by passive mass transfer in cell culture simulated in a computer model. *J. Virol.*, **86**, 10123–10137 (2012).