Published in final edited form as:

Ann Biomed Eng. 2010 December ; 38(12): 3756–3765. doi:10.1007/s10439-010-0117-0.

The Constitutive Equation for Membrane Tether Extraction

Yong Chen, Da-Kang Yao, and Jin-Yu Shao

Department of Biomedical Engineering, Washington University, Saint Louis, MO 63130

Abstract

Membrane tethers or nanotubes play a critical role in a variety of cellular and subcellular processes such as leukocyte rolling and intercellular mass transport. The current constitutive equations that describe the relationship between the pulling force and the tether velocity during tether extraction have serious limitations. Here we propose a new phenomenological constitutive equation that captures all known characteristics of nanotube formation, including nonlinearity, nonzero threshold force, and possible negative tether velocity. We used tether extraction from endothelial cells as a prototype to illustrate how to obtain the material constants in the constitutive equation. With the micropipette aspiration technique, we measured tether pulling forces at both positive and negative tether velocities. We also determined the threshold force of 55 pN experimentally for the first time. This new constitutive equation unites two established ones and provides us a unified platform to better understand not only the physiological role of tether extraction during leukocyte rolling and intercellular or intracellular transport, but also the physics of membrane tether growth or retraction.

Keywords

Membrane nanotube; leukocyte rolling; threshold force; intercellular transport; intracellular transport; micropipette aspiration

INTRODUCTION

Membrane tethers, also called membrane nanotubes, play a critical role in a variety of cellular processes such as leukocyte rolling on the endothelium^{19,23,24,30}, cancer metastasis¹⁸, intercellular organelle transport²⁰, and intracellular mass transport^{13,14,27}. To better understand membrane tether formation and extraction, many experimental techniques have been employed, including the micropipette aspiration technique (MAT), the atomic force microscopy, the optical trap, the biomembrane force probe (BFP), and the flow chamber technique^{4,10,21,23,26}. Most studies focus on characterizing the constitutive equation between the pulling force (F) and the tether velocity (U_t). To date, several constitutive equations have been proposed, two of which are used more frequently.

In an earlier study of tether extraction from passive neutrophils²³, a linear relationship was established between F and U_t with the MAT:

$$F = F_0 + 2\pi\eta_{eff}U_t, \quad (1)$$

where F_0 is the threshold force and η_{eff} is the effective viscosity. By fitting the experimental data to this equation, F_0 and η_{eff} can be determined mathematically. F_0 is the minimum force required to generate membrane flow into a tether. It is a function of membrane tension, membrane bending, and membrane-cytoskeleton adhesion energy¹¹. To our knowledge, F_0 has never been measured directly in all previous experiments. Instead, it was calculated by linear regression with Eq. 1. η_{eff} quantifies the energy dissipation due to membrane flow that, in turn, is a function of membrane viscosity, interbilayer slip, and membrane slip over the cytoskeleton¹¹. This simple linear equation has also been found to be applicable to tether extraction from lipid vesicles and several other types of cells including erythrocytes, neuronal growth cones, lymphocytes, endothelial cells, and outer-hair cells^{2,4,5,11,17,29}. However, it should be noted that the range of U_t explored in these studies is small. For example, in tether extraction from endothelial cells⁸, the range of U_t explored is from 3 to 20 $\mu\text{m/s}$; in tether extraction from leukocytes²⁵, the range of U_t explored is from 0.5 to 12 $\mu\text{m/s}$.

Besides the linear relationship described in Eq. 1, a power-law relationship that captures the shear-thinning behavior of tether extraction was also proposed on the basis of the data obtained from human neutrophils with the BFP¹⁰:

$$F = \alpha U_t^\beta. \quad (2)$$

By fitting this power-law function to the data from neutrophil tether extraction over a large range of U_t , from 0.4 to 150 $\mu\text{m/s}$, α and β were determined to be 60 $\text{pN} \cdot (\text{s}/\mu\text{m})^{0.25}$ and 0.25, respectively. For this large range of U_t , although Eq. 1 can still be applied to different intervals of U_t where linear approximation is appropriate, it is only of practical value because F_0 and η_{eff} are not constant any more. In fact, F_0 increases and η_{eff} decreases with U_t because of shear thinning, so we will name F_0 and η_{eff} the apparent threshold force and the apparent viscosity, respectively, in this study. The actual threshold force, *i.e.*, the tether pulling force when U_t is zero, will be represented by F_t .

More recently, a theoretical analysis showed that $\beta = 1/3$ for large U_t where viscous dissipation dominates membrane tension and membrane-cytoskeleton adhesion¹. Although this theoretical analysis provided a physical foundation for Eq. 2, there are other issues with this equation that remain unresolved. According to Eq. 2, F_t is equal to zero; besides, Eq. 2 is meaningless mathematically if U_t is negative. These are in direct conflict with our experimental result in this study that $F_t \neq 0$ and our simulation result that U_t may be negative during simultaneous tether extraction when leukocytes roll on the endothelium (unpublished observation). In addition, it has been shown theoretically that $F_t \neq 0$ because of membrane tension, membrane bending, and membrane-cytoskeleton adhesion^{1,11}. It has also been shown experimentally that $F_t \neq 0$ if an extracted tether is held at constant length^{10,28}. The relationship between F and U_t when U_t is negative describes how an extracted tether shortens when F is less than F_t . With their derivation for small U_t , Brochard-Wyart et al. predicted correctly that $F_t \neq 0$, but their analysis cannot be applied when U_t is negative¹. This is because, according to their model, $U_t = 0$ when $F = 0$. This prediction is clearly not true because it is a known fact that an extracted tether shortens quickly back to the cell body once F is removed, *i.e.*, U_t is negative when $F = 0$ ⁹.

In this study, we propose a new phenomenological constitutive equation in the form of

$$F = \alpha (U_t - U_0)^\beta, \quad (3)$$

where U_0 is the tether velocity when $F = 0$. Although Eq. 3 can be used to describe tether extraction from all other cell types, subcellular organelles, and lipid vesicles, we will only illustrate how to obtain α , β , and U_0 with tether extraction from human umbilical vein endothelial cells (HUVECs) as an example. By pulling single tethers with the MAT, we determined the relationship between F and U_t when U_t was negative. We also measured F_t directly and experimentally for the first time. When U_t was positive, we showed experimentally that Eq. 1 was still valid in small intervals of U_t and η_{eff} decreased while F_0 increased with increasing U_t , in agreement with the shear thinning behavior of membrane tethers. Equation 3 unites Eqs. 1 and 2 and it allows us to better model and understand tether extraction or retraction because it captures all known characteristics of tether extraction, including nonlinearity, nonzero threshold force, and possible negative tether velocity.

MATERIALS AND METHODS

Cell Culture and Preparation

HUVECs (Cambrex Biosciences, Walkersville, MD) were cultured in 6-well plates with endothelial cell medium-2 (Cambrex Biosciences) at 37°C in 5% CO₂. For experiments with suspended HUVECs, the confluent cells in one well were detached with 5 mM EDTA (Sigma, Saint Louis, MO), washed with 0.1% bovine serum albumin (BSA), and then transferred into the experimental chamber with CO₂-independent medium for immediate use (Invitrogen Corporation, Carlsbad, CA). For experiments with attached HUVECs, a cell-culture-treated coverslip (Fisher Scientific, Hampton, NH) was prepared and mounted on the inner side wall of a special chamber in advance. After the coverslip was firmly adhered, suspended cells were cultured on the coverslip and the whole chamber was then kept in the incubator for 12 hours for the cells to attach and spread. Prior to experiment, the chamber was removed from the incubator, washed, and refilled with CO₂-independent medium. To study the effect of actin cytoskeleton on tether extraction, a final concentration of 5 μ M latrunculin A (Calbiochem, La Jolla, CA) was used in the chamber and the experiments were performed after the cells had been incubated for 20 min at room temperature.

Micropipette Aspiration Technique: Preparation and Operation

The preparation and assembly of the MAT were described in detail previously. Briefly, spherical latex beads (~8 μ m in diameter; Sigma) coated with goat-anti-mouse antibodies were employed as the force transducer of the MAT. The beads were washed twice in PBS and then incubated with mouse anti-human antibodies (anti-CD31, R&D Systems, Minneapolis, MN) for 1 hour at 37°C. Prior to experiment, 20- μ l beads were taken out, washed twice in PBS, and then re-suspended in CO₂-independent medium. For surface-attached HUVECs, a glass micropipette whose diameter was almost identical to the bead diameter was prepared and used to contain the bead (Fig. 1A); for suspended HUVECs, another micropipette was set up to restrain the cell with a small aspiration pressure (Fig. 1B). The narrow opening of the micropipette was filled with 1% BSA and the rest of it was backfilled with PBS. The end of the pipette was connected to a water reservoir through Tygon tubing. The bead could move freely inside the pipette and its velocity is mainly determined by the pressure drop generated between the reservoir and the pipette.

A modification was made to the manometer of the MAT to enhance its capability in applying variable forces on cells and molecules³. A motorized system with two vertical translational stages (one motorized (Physik Instrumente, Auburn, MA. Model M-501.1PD) and the other manual) assembled in series was used to control the height of the water reservoir. By controlling the movement of the motorized stage with a program in LabView (National Instruments, Austin, TX), we could apply an increasing pressure or any pressure pattern in the micropipette. In this work, two different pressure patterns were programmed

based on two different purposes. In both programs, a 5V electric signal was sent out through the output function of the DAQ board to record the instant when the stage started to move in each contact event. When the speed of the stage was less than or equal to 200 $\mu\text{m/s}$, this electric signal was sent to a multiplexer and a voltage stamp of 5 V was then printed on the monitor along with the image signal transmitted from the analog camera (Fig. 1). The whole experiment was recorded onto a DVD with a Sony DVD recorder. When the stage speed was larger than 200 $\mu\text{m/s}$, a high-speed digital camera (Vision Research, Wayne, NJ) was utilized for the image acquisition. A sampling rate between 100 and 500 frames/s was chosen based on motorized stage speed. The previous electric signal was used to trigger the recording of this high-speed digital camera.

Data Analysis

For the experiments performed at the lower stage speed (less than or equal to 200 $\mu\text{m/s}$), all experimental data recorded with the Sony DVD recorder was transmitted to the computer through a monochrome frame grabber. Each contact event between the bead and the cell was saved in an individual movie in TIF format. For the experiments performed at the higher stage speed (larger than 200 $\mu\text{m/s}$), each contact event was transmitted directly from the digital camera to the computer and then saved in an individual TIF movie. The displacement of the bead was tracked with a home-written program in MATLAB. The tracking algorithm is based on the method developed by Gelles et al.⁶ and it has a tracking resolution of ~ 5 nm. Half of the bead image in the first frame was chosen as the kernel for the tracking and the result was saved in an ASCII file. The data in this file were then imported into another MATLAB program having a custom-designed and -programmed graphic user interface. To calculate the bead velocity, a linear fit was performed on nine consecutive points and the slope obtained from the fitting was the velocity at the center point. When the aspiration pressure is not zero, the force acting on the bead (Fig. 1) can be calculated from²⁵

$$F = \pi R_p^2 \Delta p \left(1 - \frac{4}{3} \bar{\varepsilon} \right) \left(1 - \frac{U_t}{U_f} \right), \quad (4)$$

where Δp is the suction pressure at the time point, R_p is the radius of the micropipette, U_t is the bead velocity when it is moving freely under Δp , and

$$\bar{\varepsilon} = (R_p - R_b) / R_p, \quad (5)$$

where R_b is the radius of the bead.

When the pressure is zero, the pulling force can be calculated from^{3,23}

$$F = \frac{\Delta p - \frac{\mu U_t}{R_p} \left[\left(\frac{4\sqrt{2}\pi}{\bar{\varepsilon}^{1/2}} - 32 + \frac{2\sqrt{2}\pi}{3} \bar{\varepsilon}^{1/2} \right) + \frac{8(L_{eq} - 2R_b)}{R_p} \left(1 - \frac{4}{3} \bar{\varepsilon} + \frac{16}{9} \bar{\varepsilon}^2 \right) \right]}{\pi R_p^2 \left[\left(1 + \frac{4}{3} \bar{\varepsilon} + \frac{16}{9} \bar{\varepsilon}^2 \right) + \frac{8(L_{eq} - 2R_b)}{R_p} \left(\frac{2\sqrt{2}}{9\pi} \bar{\varepsilon}^{5/2} \right) \right]}, \quad (6)$$

where μ is the viscosity and L_{eq} is the equivalent length of the micropipette, which can be calculated from

$$L_{eq} = \frac{R_p}{8 \left(1 - \frac{4}{3}\varepsilon + \frac{16}{9}\varepsilon^2\right)} \left[\frac{\Delta p R_p}{\mu U_f} - \left(\frac{4\sqrt{2}\pi}{\varepsilon^{1/2}} - 32 + \frac{2\sqrt{2}\pi}{3}\varepsilon^{1/2} \right) \right] + 2R_b. \quad (7)$$

The diameter of the micropipette ($2R_p$) was determined with differential interference contrast microscopy and the bead diameter ($2R_b$) was determined with bright field microscopy. Both values were then divided by their corresponding correction factors as described before²⁵. In this study, the gap between the bead and the micropipette was $\sim 0.2 \mu\text{m}$.

RESULTS

From our proposed new constitutive equation, we can easily obtain

$$F_t = \alpha(-U_0)^\beta. \quad (8)$$

Therefore, if F_t can be measured experimentally, it can be used to examine the reliability of the parameters determined in Eq. 3. With the MAT, we directly-measured F_t for tether extraction from both suspended and attached HUVECs. We also determined the relationship between F and U_t at both negative and positive tether velocities. The magnitude of the force applied using the MAT is determined by the aspiration pressure in the micropipette that contains the force transducer. In our studies, two different pressure patterns were employed for two different purposes.

Shear-Thinning of HUVEC Tether Extraction

Although tether extraction from HUVECs has been studied before, it was only studied for a small range of U_t where Eq. 1 can be applied. To obtain the parameters in the new constitutive equation for tether extraction from HUVECs, we first extracted tethers at increasing velocities by increasing pulling forces with the MAT. In order to apply an increasing force, we programmed a motorized manometer to rise at a constant speed and applied an increasing aspiration pressure in the right micropipette as shown in Fig. 1. In a typical contact event between the bead and the cell, if there was no adhesive bond formed between them at the end of the contact, the bead would move downstream in a parabolic trajectory, *i.e.*, at a constant acceleration³. On the other hand, if a bond was formed between the bead and the cell, two different regimes were identified before the final failure of this bond. Figure 2A shows the typical displacement of an anti-CD31-coated bead acquired at the manometer speed of $100 \mu\text{m/s}$ (equivalent to an increasing pressure of $1 \text{ pN}/\mu\text{m}^2/\text{s}$). In the first regime, a short cell surface protrusion of several hundred nanometers was observed before the onset of tether extraction (at $\sim 3 \text{ s}$). The subject of surface protrusion will be dealt with in a separate study and we will focus on tether extraction in this paper. In the second regime, a tether was extracted for more than $30 \mu\text{m}$ before the adhesion was ruptured at about 9.5 s . In the course of tether extraction, the tether velocity (U_t) increased from $1 \mu\text{m/s}$ to more than $10 \mu\text{m/s}$, while the force (F) also increased from about 60 pN to 90 pN . By plotting F against U_t (Fig. 2B), we found a linear relationship between them in the current velocity range. The origin of the sawtooth-like fluctuation in F is not completely understood, but it is likely due to the experimental noise in the displacement data³.

By varying the increasing pressure, we also investigated tether extraction with larger ranges of U_t . The range of U_t is mainly determined by the increasing pressure and the lifetime of the adhesive bond between the cell and the bead. For the tethers extracted with the same

increasing pressure, the ranges of U_t were usually similar. Figure 3 shows the relationship between F and U_t for single tether extraction obtained at two different increasing pressures. The average tether velocities obtained at the increasing pressures of 0.25 and 30 pN/ $\mu\text{m}^2/\text{s}$ were 3.49 and 39.26 $\mu\text{m}/\text{s}$, respectively. We found that, as long as tethers were obtained with the same increasing pressure, the relationship between F and U_t for all the tethers can be described by Eq. 1 very well. However, both F_0 and η_{eff} yielded from linear regression were markedly different between the two increasing pressures (Fig. 3).

Figure 4 presents all the values of F_0 and η_{eff} obtained from different increasing pressures. With the method described in Fig. 3, both F_0 and η_{eff} were calculated for all the tethers obtained at each individual increasing pressure, and so was the average U_t . The dependence of F_0 and η_{eff} on U_t is shown in Figs. 4A&B where each point was obtained from 6–21 tethers extracted from 2 to 11 cells at the same increasing pressure. More than ten different increasing pressures were used in the range of 0.1 to 50 pN/ $\mu\text{m}^2/\text{s}$. Altogether around 300 tethers were extracted from 135 cells. For both suspended and attached HUVECs, F_0 increased from about 30 pN to 80 pN when U_t increased from 2 to 40 $\mu\text{m}/\text{s}$ (Fig. 4B). On the other hand, η_{eff} decreased as U_t increased (Fig. 4A). At the increasing pressure of 0.02 pN/ $\mu\text{m}^2/\text{s}$, η_{eff} was about 1.5 pN·s/ μm , which is more than three times of the value obtained previously⁸. However, at the maximum velocity of 40 $\mu\text{m}/\text{s}$, η_{eff} was only 0.2 pN·s/ μm , which was about 40% of the value obtained previously. The increase in F_0 and the decrease in η_{eff} simply demonstrate the shear-thinning behavior of tether extraction from endothelial cells, as has been found in neutrophils¹⁰.

The shear-thinning behavior of tether extraction is dependent on the cytoskeletal integrity. To evaluate how actin filaments influence shear thinning, we treated both suspended and attached cells with 5 μM latrunculin A for 20 min and pulled out tethers from these treated cells with the MAT. Four different increasing pressures from 0.2 to 10 pN/ $\mu\text{m}^2/\text{s}$ were chosen and a total of 55 tethers were extracted from 40 cells. Compared to the results obtained from native cells (Fig. 4A&B), the dependence of both F_0 and η_{eff} on U_t vanished after the actin cytoskeleton was disrupted (Fig. 4C&D). We also examined tether extraction from both TNF- α -stimulated and IL-1 β -stimulated cells and the results showed that the shear-thinning behavior was independent of TNF- α or IL-1 β stimulation of endothelial cells (data not shown).

Direct Measurement of F_t and the Relationship between F and U_t when $U_t < 0$

Using a different pressure pattern, we directly-measured F_t experimentally for the first time. In this experiment, we first applied a constant pressure to extract a tether at a positive constant velocity. Then we gradually decreased the pressure until it became zero, which caused tether velocity to decrease from a positive value to a negative one. From the particular pressure at which U_t was zero, we calculated the corresponding pulling force, which should be F_t . Figure 5 shows one such experiment obtained with a suspended HUVEC. As shown in the figure, the pressure was first ramped up quickly to a constant pressure of 4 pN/ μm^2 , and then decreased at a speed of 0.5 pN/ $\mu\text{m}^2/\text{s}$ after tether extraction was initiated. As a result, the tether velocity initially jumped to about 5.4 $\mu\text{m}/\text{s}$, and then gradually decreased to about zero at 6.4 s. At this moment, the pulling force was calculated to be about 48 pN. As the pressure continued to decrease, the tether started to retract at around 7 s. At about 10.5 s, the pressure was ramped back up to 4 pN/ μm^2 and the tether grew at a constant velocity of about 5.4 $\mu\text{m}/\text{s}$ again before the adhesion was ruptured around 13 s. The bead displacement after 10.5 s clearly shows that only one tether was between the cell and the bead during the whole process.

This measurement was also repeated with attached HUVECs. For both attached and suspended HUVECs, we used two different speeds to decrease the pressure. A summary of

all forces, as well as their standard deviations, is shown in Table 1. For the four groups studied, a total of 72 tethers were extracted from 28 cells. No significant difference was found in the force magnitude among the four groups ($p > 0.05$), which indicates that the threshold force for tether extraction from endothelial cells is independent of cell attachment state and tether deceleration speed. Among all the four groups, the average threshold force is 55 ± 10 pN (mean $\pm$ standard deviation).

In addition to F_t , the relationship between F and U_t when U_t is negative can also be obtained from this experiment. As shown in Fig. 5, U_t started to be negative at about 6.4 s and continued its decrease until about 10.5 s. The corresponding pulling forces during this time window can be calculated and used for establishing the relationship between F and U_t . For the case shown in Fig. 5, U_t was -1.4 $\mu\text{m/s}$ when the pressure reached zero and the corresponding pulling force was around 40 pN. For all the tethers studied in this set of experiments, they retracted at a negative U_t once the pulling force fell below F_t (data not shown).

The Constitutive Equation for Tether Extraction

Neither Eq. 1 nor Eq. 2 alone is adequate to accommodate shear thinning, nonzero threshold force, and negative tether velocity of tether extraction. Therefore, Eq. 3 was proposed as the constitutive equation for tether extraction from endothelial cells. To obtain α , β , and U_0 in Eq. 3, we plotted all the tether data with either positive or negative U_t in Fig. 6 and fitted them to Eq. 3. Because of our direct experimental measurement of F_t , Eq. 8 provides an additional means to examine the reliability of α , β , and U_0 determined in Eq. 3. Altogether more than 40000 data points were obtained from both attached and suspended cells in our shear-thinning experiments and more than 14000 points were obtained from our threshold force measurements. For both suspended and attached HUVECs, the data from these two different experiments (shear thinning and negative tether velocity) match very well with each other around zero tether velocity. This is a strong indication that the constitutive equation is independent of how pressures were applied (increasing or decreasing) in our experiments.

As shown in Figs. 6A&B, our new constitutive equation fits the data very well and α , β , and U_0 can be obtained. With these parameters and Eq. 3, we calculated F_0 and η_{eff} at different U_t , which agree very well with what is shown in Figs. 4A&B (data not shown). For attached and suspended HUVECs, the threshold forces calculated with Eq. 8 are 55 and 51 pN, respectively, which are very close to our direct measurements (Table 1). Further analysis verified that the data points obtained at negative tether velocities are very important in determining α , β , and U_0 . After the deletion of these points (around 1600 points for attached cells and 4200 points for suspended cells), the value of U_0 obtained would decrease 48% for attached cells and increase 92% for suspended cells. This further shows the importance of measuring the relationship between F and U_t when U_t is negative.

DISCUSSION

In this study, we proposed a new constitutive equation for tether extraction from cells, subcellular organelles, or lipid vesicles. Although phenomenological in nature, this new equation covers a large range of tether velocities including negative ones and it captures all known characteristics of tether extraction. It also unites Eqs. 1 and 2 in the following fashion:

$$F = \begin{cases} \alpha(-U_0)^\beta + \alpha\beta(-U_0)^{\beta-1}U_t = F_0 + 2\pi\eta_{eff}U_t & \text{when } |U_t| \ll -U_0 \\ \alpha U_t^\beta & \text{when } |U_t| \gg -U_0 \end{cases} \quad (9)$$

This provides us a means to relate α , β , and U_0 to cellular mechanical properties through F_0 and η_{eff} , but their physical interpretations remain a challenge. One limitation of our proposed equation is that it cannot be applied to the cases where $F < 0$. We illustrated how to obtain the material constants in the constitutive equation by pulling out membrane tethers from HUVECs with the MAT. We employed two different pressure patterns for two different purposes. In our first set of experiments, the shear-thinning behavior in tether extraction was clearly demonstrated from our estimation of F_0 and η_{eff} at different pulling velocities; in our second set of experiments, F_t and the relationship between F and U_t when U_t is negative were directly measured experimentally. Both suspended and surface-attached cells were studied and no obvious difference between these two groups was found, consistent with our previous observations ².

There is no doubt that a nonzero threshold force exists for tether extraction. This has been shown experimentally in this study and theoretically in others ^{1,11}. From a mechanical standpoint, even at zero tether velocity, a pulling force is needed because of membrane tension, membrane bending, and membrane-cytoskeleton adhesion. Even for lipid vesicles where there is no membrane-cytoskeleton adhesion, the threshold force is clearly shown to exist ⁵. The magnitude of the threshold force measured here is very close to what was calculated with Eq. 1 in previous experiments ⁸ or with Eq. 8 in this study, and it is also independent of the cell attachment state as found in previous studies.

At the moment when simultaneous tethers are extracted during leukocyte rolling, the force exerted by the blood flow on the adhesive bond is larger than both threshold forces of tether extraction from leukocytes and endothelial cells. Afterwards this force continuously decreases because of tether extraction ⁹. Eventually, the force will fall below the larger threshold force, but remain above the smaller one. At this moment, one of the tethers will shorten while the other will continue to lengthen, resulting in an overall positive tether growth. Although Eq. 1 can be used to describe tether extraction at negative tether velocities, its reliability at negative U_t is seriously in doubt due to the shear-thinning behavior of tether extraction. The physiological significance of having a constitutive equation that can be applied at negative U_t is evident in the constant formation and retraction of membrane nanotubes observed in the endoplasmic reticulum, causing the construction and rearrangement of the network ¹⁵. In addition, tether retraction occurs when the adhesive bond is ruptured between the leukocyte and endothelial cell during leukocyte rolling and it also occurs during simultaneous tether extraction as mentioned above.

The technique used in our study, the MAT, has been applied to many studies including membrane tether extraction from single cells, surface receptor expression, and single molecule mechanics ^{16,22,23}. The advantages of using the MAT to study tether extraction lie in several aspects. Firstly, tether extraction can be studied in a large range of U_t , including negative ones. Secondly, it can be used to study both tether extraction from single cells (suspended and attached cells) and simultaneous tether extraction from a spherical cell and an attached cell ^{2,7-9}. Thirdly, it can apply a large range of force from several piconewtons up to hundreds of nanonewtons. Earlier studies of tether extraction using the MAT were limited by the manually-controlled manometer. Therefore, only constant pressure was applied, which causes tether extraction merely at constant velocities. Recently, we modified the MAT by replacing the manually-controlled manometer with a computer-controlled system ³. This new modification allowed us to conduct the experiments in this study.

In our experiments with the MAT, membrane receptors were used as the force handle to extract membrane tethers. Previous studies have shown that mechanics of tether extraction is independent of the type of receptor that is targeted in experiments ^{2,8}. Therefore, only one type of mouse anti-human antibody (anti-CD31) was used to coat the latex beads in the

present study. In addition, tether extraction from endothelial cells is found to be independent of cell lineage ². Therefore, the constitutive equation we obtained here should be applicable to tether extraction with other receptors from other lineages of endothelial cells. Moreover, we also fitted this constitutive equation to tether extraction from neutrophils ^{10,23}, neuronal growth cones ¹¹, red blood cells ¹², and outer hair cells ¹⁷ and obtained α , β , and U_0 for each cell type (data not shown). The minimum correlation coefficient obtained from these fittings is 0.84, which demonstrates that this new constitutive equation can be indeed used to describe tether extraction from these cells. However, without any data at negative tether velocity, the parameters obtained are not very reliable, especially U_0 .

We have shown here that mechanics of tether extraction does not depend on whether a tether is extracted under a constant or an increasing velocity. This conclusion is further supported by two pieces of evidence. Firstly, the data points obtained in this study (Fig. 6) are in excellent agreement with our previous findings at the tether velocities from 3 to 20 $\mu\text{m/s}$ ⁸. Secondly, we used another pressure pattern where an increasing pressure was followed by a constant pressure to prove this hypothesis (data not shown). With this pressure pattern, a tether was first extracted at an increasing U_t and then kept growing at a constant U_t . When U_t was increasing, a linear relationship between F and U_t was obtained, which is similar to the result shown in Fig. 2B. When U_t was a constant, only one pulling force was obtained and this force matches very well with the linear relationship established earlier. Therefore, this is a direct demonstration that the acceleration of U_t does not affect the constitutive equation between F and U_t .

In conclusion, we studied both tether extraction and retraction from HUVECs using the MAT and established a new constitutive equation that can describe both phenomena in a large range of tether velocity. This equation can also be applied to tether extraction from other eukaryotic cells and subcellular organelles in different cellular processes. Therefore, it provides us a unified platform to better understand membrane tether growth and retraction in the future.

Acknowledgments

This work was supported by NIH (R01 HL069947 and R21/R33 RR017014).

REFERENCES

1. Brochard-Wyart F, Borghi N, Cuvelier D, Nassoy P. Hydrodynamic narrowing of tubes extruded from cells. *Proc. Natl. Acad. Sci. U. S. A* 2006;103:7660–7663. [PubMed: 16679410]
2. Chen Y, Girdhar G, Shao JY. Single membrane tether extraction from adult and neonatal dermal microvascular endothelial cells. *Am. J. Physiol. Cell Physiol* 2007;292:C1272–C1279. [PubMed: 17079334]
3. Chen Y, Liu B, Xu G, Shao J-Y. Validation, in-depth analysis, and modification of the micropipette aspiration technique. *Cell. Mol. Bioeng* 2009;2:351–365. [PubMed: 20333318]
4. Dai J, Sheetz MP. Mechanical properties of neuronal growth cone membrane studied by tether formation with laser optical tweezers. *Biophys. J* 1995;68:988–996. [PubMed: 7756561]
5. Evans E, Yeung A. Hidden dynamics in rapid changes of bilayer shape. *Chem. Phys. Lipids* 1994;73:39–56.
6. Gelles J, Schnapp BJ, Sheetz MP. Tracking kinesin-driven movements with nanometer-scale precision. *Nature* 1988;331:450–453. [PubMed: 3123999]
7. Girdhar G, Chen Y, Shao JY. Double tether extraction from human umbilical vein and dermal microvascular endothelial cells. *Biophys. J* 2007;92:1035–1045. [PubMed: 17098792]
8. Girdhar G, Shao JY. Membrane tether extraction from human umbilical vein endothelial cells and its implication in leukocyte rolling. *Biophys. J* 2004;87:3561–3568. [PubMed: 15339799]

9. Girdhar G, Shao JY. Simultaneous Tether Extraction from Endothelial Cells and Leukocytes: Observation, Mechanics and Significance. *Biophys. J* 2007;93:4041–4052. [PubMed: 17704170]
10. Heinrich V, Leung A, Evans E. Nano- to microscale dynamics of P-selectin detachment from leukocyte interfaces. II. Tether flow terminated by P-selectin dissociation from PSGL-1. *Biophys. J* 2005;88:2299–2308. [PubMed: 15653735]
11. Hochmuth RM, Shao JY, Dai J, Sheetz MP. Deformation and flow of membrane into tethers extracted from neuronal growth cones. *Biophys. J* 1996;70:358–369. [PubMed: 8770212]
12. Hwang WC, Waugh RE. Energy of dissociation of lipid bilayer from the membrane skeleton of red blood cells. *Biophys. J* 1997;72:2669–2678. [PubMed: 9168042]
13. Koster G, VanDuijn M, Hofs B, Dogterom M. Membrane tube formation from giant vesicles by dynamic association of motor proteins. *PNAS* 2003;100:15583–15588. [PubMed: 14663143]
14. Leduc C, Campas O, Zeldovich KB, Roux A, Jolimaître P, Bourel-Bonnet L, Goud B, Joanny JF, Bassereau P, Prost J. From the Cover: Cooperative extraction of membrane nanotubes by molecular motors. *PNAS* 2004;101:17096–17101. [PubMed: 15569933]
15. Lee C, Chen LB. Dynamic behavior of endoplasmic reticulum in living cells. *Cell* 1988;54:37–46. [PubMed: 3383243]
16. Levin JD, Ting-Beall HP, Hochmuth RM. Correlating the kinetics of cytokine-induced E-selectin adhesion and expression on endothelial cells. *Biophys. J* 2001;80:656–667. [PubMed: 11159434]
17. Li Z, Anvari B, Takashima M, Brecht P, Torres JH, Brownell WE. Membrane tether formation from outer hair cells with optical tweezers. *Biophys. J* 2002;82:1386–1395. [PubMed: 11867454]
18. McCarty OJT, Mousa SA, Bray PF, Konstantopoulos K. Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions. *Blood* 2000;96:1789–1797. [PubMed: 10961878]
19. Ramachandran V, Williams M, Yago T, Schmidtke DW, McEver RP. Dynamic alterations of membrane tethers stabilize leukocyte rolling on P-selectin. *PNAS* 2004;101:13519–13524. [PubMed: 15353601]
20. Rustom A, Saffrich R, Markovic I, Walther P, Gerdes HH. Nanotubular Highways for Intercellular Organelle Transport. *Science* 2004;303:1007–1010. [PubMed: 14963329]
21. Schmidtke DW, Diamond SL. Direct observation of membrane tethers formed during neutrophil attachment to platelets or P-selectin under physiological flow. *J. Cell Biol* 2000;149:719–729. [PubMed: 10791984]
22. Shao JY, Hochmuth RM. Mechanical anchoring strength of L-selectin, β_2 integrins and CD45 to neutrophil cytoskeleton and membrane. *Biophys. J* 1999;77:587–596. [PubMed: 10388783]
23. Shao JY, Hochmuth RM. Micropipette suction for measuring piconewton forces of adhesion and tether formation from neutrophil membranes. *Biophys. J* 1996;71:2892–2901. [PubMed: 8913626]
24. Shao JY, Ting-Beall HP, Hochmuth RM. Static and dynamic lengths of neutrophil microvilli. *PNAS* 1998;95:6797–6802. [PubMed: 9618492]
25. Shao JY, Xu J. A modified micropipette aspiration technique and its application to tether formation from human neutrophils. *J. Biomech. Eng* 2002;124:388–396. [PubMed: 12188205]
26. Sun M, Northup N, Marga F, Huber T, Byfield FJ, Levitan I, Forgacs G. The effect of cellular cholesterol on membrane-cytoskeleton adhesion. *J. Cell Sci* 2007;120:2223–2231. [PubMed: 17550968]
27. White J, Johannes L, Mallard F, Girod A, Grill S, Reinsch S, Keller P, Tzschaschel B, Echard A, Goud B, Stelzer EHK. Rab6 Coordinates a Novel Golgi to ER Retrograde Transport Pathway in Live Cells. *J. Cell Biol* 1999;147:743–760. [PubMed: 10562278]
28. Xu, G. *Mechanics of Single Microvilli and Double Tethers of Human Neutrophils and Lymphocytes*. Saint Louis, MO: Washington University; 2006.
29. Xu G, Shao JY. Double tether extraction from human neutrophils and its comparison with CD4+ T-lymphocytes. *Biophys. J* 2005;88:661–669. [PubMed: 15475589]
30. Yago T, Leppanen A, Qiu H, Marcus WD, Nollert MU, Zhu C, Cummings RD, McEver RP. Distinct molecular and cellular contributions to stabilizing selectin-mediated rolling under flow. *J. Cell Biol* 2002;158:787–799. [PubMed: 12177042]

FIGURE 1.

Two video micrographs showing the setup of the micropipette aspiration technique. An antibody-coated bead contained in a micropipette was used as the force transducer to interact with an attached (A) or suspended (B) HUVEC. A voltage stamp of 5V was used to indicate the motion status of the motorized manometer.

FIGURE 2.

A typical case in a tether extraction experiment acquired at an increasing pressure of 100 pN/ $\mu\text{m}^2/\text{s}$. (A) The bead displacement shows that a tether was pulled out from 3.5 s to 9.5 s with an increasing velocity. During this process, the pulling force also increased over time. (B) The relationship between the pulling force (F) and the tether velocity (U_t). The solid line represents a linear fit to all the data points, and the corresponding fitted equation and correlation coefficient are also shown.

FIGURE 3.

The relationship between the pulling force and the tether velocity for tether extraction obtained at two speeds of increasing pressure. (A) Six tethers were extracted from four suspended HUVECs at the increasing pressure of $0.25 \text{ pN}/\mu\text{m}^2/\text{s}$. Overall, around 1300 data points were plotted and the average tether growth velocity was $3.49 \mu\text{m/s}$. (B) 12 tethers were extracted from three attached HUVECs at the increasing pressure of $30 \text{ pN}/\mu\text{m}^2/\text{s}$. Around 1700 data points were plotted and the average tether growth velocity was $39.26 \mu\text{m/s}$. The solid lines represent a linear fit to all the data points, which determines apparent F_0 and η_{eff} for the tethers obtained at the same increasing pressure. The corresponding fitted equations and correlation coefficients are also shown.

FIGURE 4.

Dependence of apparent F_0 and η_{eff} on tether velocity. At each increasing pressure, F_0 , η_{eff} , and average tether velocity were determined as shown in Fig. 3. For native cells (both attached and suspended), apparent η_{eff} (A) and apparent F_0 (B) are both dependent on tether velocity, which directly demonstrates the shear-thinning behavior of tether extraction from endothelial cells. However, this dependence almost vanished after the treatment with 5 μ M latrunculin A (C&D). The error bars in the vertical direction represent the 95% confidence intervals of apparent η_{eff} (A&C) and apparent F_0 (B&D), while the error bars in the horizontal direction represent the standard deviations of the tether velocities used for estimating η_{eff} and F_0 . It should be noted that these standard deviations do not represent how accurately the tether velocities were measured.

FIGURE 5.

Direct measurement of F_t . According to the bead displacement (solid line), the bead reached zero velocity at 6.4 s. At this moment, the pulling force (i.e., F_t) was about 48 pN, which was calculated from the applied suction pressure (dotted line).

(A)

(B)

FIGURE 6.

The correlation between the pulling force and the tether velocity for tether extraction from HUVECs obtained at both positive and negative tether velocities. Overall, 21749 points from 144 tethers were obtained from attached cells (A), and 32685 points from 216 tethers were obtained from suspended cells (B). The data were fitted to Eq. 3 and the fitted line is also shown. For attached cells, α is $27.68 \pm 0.82 \text{ pN} \cdot (\text{s}/\mu\text{m})^{0.45}$, β is 0.45 ± 0.01 and U_0 is $-4.63 \pm 0.22 \mu\text{m/s}$, while for suspended cells, α is $22.25 \pm 0.82 \text{ pN} \cdot (\text{s}/\mu\text{m})^{0.46}$, β is 0.46 ± 0.01 and U_0 is $-6.17 \pm 0.30 \mu\text{m/s}$. The values for all the parameters are expressed as means $\pm$ the 95% confidence intervals.

TABLE 1

Summary of F_t measured at zero tether velocity.

Decreasing pressure (pN/ $\mu\text{m}^2/\text{s}$)	Attached	Suspended
0.5	59.1 ($N = 10$)	53.1 ($N = 27$)
0.75	56.5 ($N = 14$)	55.4 ($N = 21$)
Total (mean $\pm$ SD)	57.6 $\pm$ 5.5	54.1 $\pm$ 11.3

N denotes the number of tethers extracted at each condition. Both suspended and attached HUVECs were studied at each condition and two different speeds of decreasing pressure were used to gradually decrease the aspiration pressure to zero. No significant difference was found among the four different groups ($p > 0.05$).