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Detachment-associated changes in lipid rafts of senescent human fibroblasts

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Abstract

We characterized the effects of in vitro cellular aging on constituents of lipid rafts in human diploid fibroblasts, TIG-1. Cholesterol recovery from lipid rafts of senescent cells was decreased by the detaching treatment, while the decrease was far less obvious in young cells. A probe that binds selectively to cholesterol in lipid rafts revealed that the amount of lipid rafts on the cell surface decreased in senescent cells upon cell detachment. Accompanying this change was the release of the raft-associated molecules caveolin and Fyn from lipid rafts upon cell detachment, suggesting a detachment-associated disorganization of lipid rafts in senescent cells. In addition, our observations showing differential sensitivities of lipid rafts from young and senescent cells to detaching treatment indicate a caution in how to detach cells. Particular attention needs to be paid to interpreting the results when lipid rafts are prepared from mechanically detached cells under detergent-free conditions.

Keywords: Senescence; Lipid raft; Adhesion; Fibroblast; Caveolin; Cholesterol

Mounting evidence suggests that cholesterol- and sphingolipid-rich microdomains (lipid rafts) exist in the plasma membrane [1–4]. Specific proteins, including caveolins, flotillins, Src-family kinases, and glycosylphosphatidylinositol-linked proteins, assemble in these membrane domains where they play a role in signal transduction and many other cellular events [1,3–5]. Changes in cholesterol content, either by inhibition of its biosynthesis or by its removal from the plasma membrane, affect the localization of proteins associated with lipid rafts, and thus affect their function [3–5], which suggests that cholesterol plays an essential role in the structural maintenance and function of lipid rafts. Detergent-resistant membranes (DRMs) have been suggested to originate from the cholesterol- and sphingolipid-rich l_o phase that resists extraction due to its tight lipid packing [2], and thus DRMs have been used for the biochemical characterization of lipid rafts in many studies. In recent years, detergent-free preparation methods have also been used for this purpose [4,6,7].

Aging causes a decline and dysregulation of membrane-mediated signal transduction in many types of cells and organs [8,9]. To understand the mechanism of age-related dysfunction in signal transduction, age-dependent changes in lipid raft-mediated signaling have been studied in recent years [10–14]. Lipid rafts participate in T-cell receptor signaling [15], and some reports have suggested that age-dependent changes in the components of lipid rafts are responsible for the decline in T-cell receptor signaling in mouse [10] and human T cells [11]. In adherent cells, lipid rafts have been proposed to mediate an adherent signal to the cell interior through an integrin-mediated signaling pathway, and thus to participate in anchorage-dependent cell growth [16,17]. Studies on in vitro cellular aging have shown that senescent fibroblasts enter irreversible growth

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arrest and exhibit hypo-responsiveness to stimulation with growth factors [9]. Recent reports have suggested that caveolin-1, a component of lipid rafts, plays a key role in the growth arrest and other senescent phenotypes of human diploid fibroblasts [12,13,18,19]. The over-expression of caveolin-1 induces premature senescence [18], and a reduction in the level of caveolin-1 by its antisense oligonucleotides and small interfering RNA reverses the senescent phenotype [19]. Thus, caveolin and caveolin-containing lipid rafts are implicated in cellular aging.

Focusing on cholesterol as a major component of lipid rafts, we have prepared a probe, BCθ, a non-cytolytic derivative of θ-toxin (perfringolysin O), for detecting cholesterol in lipid rafts [20–23]. We demonstrated that cell-bound BCθ is predominantly recovered in detergent-insoluble low-density membrane fractions (raft fractions) [21,22]. Recent biochemical and cytochemical studies have shown that BCθ is an excellent probe for detecting cholesterol-enriched membrane microdomains [21,24,25].

By using BCθ we have been characterizing the effects of in vitro cellular senescence on lipid rafts of human diploid fibroblasts, TIG-1 cells. We previously reported that the levels of cholesterol and some other raft marker molecules recovered in the raft fractions of senescent cells were significantly lower than in young cells [14]. However, we recently noticed that the difference between young and senescent cells is highly dependent on the preparation conditions, that is, how the cells are detached from the culture dishes. In this report, we examine how the preparation methods influence the composition/organization of lipid rafts, and show that lipid rafts in senescent cells are much more sensitive to the detaching treatment than those in young cells. The level of cell surface lipid rafts decreases remarkably upon mechanical detachment of senescent cells, while the change is far less obvious in young cells. Accompanying this decrease, some, but not all, lipid raft components, i.e., cholesterol, caveolin, Fyn, and GM1, are released from lipid rafts upon the detachment of senescent cells. These findings indicate differential sensitivities of lipid rafts from senescent and young cells to detaching treatments. Our observations indicate a caution for the preparation methods of lipid rafts, since the mechanical detachment of intact cells from culture dishes might cause changes in lipid rafts.

Materials and methods

Materials. Anti-θ-toxin was produced as described previously [26]. Antibody against caveolin was purchased from Transduction Laboratories (Lexington, KY, USA). Anti-flotillin-1 was from BD Bioscience (San Jose, CA, USA). Anti-Fyn was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cholera toxin B subunit-peroxidase conjugate, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and pepstatin A were from Sigma–Aldrich (St. Louis, MO, USA). Leupeptin was from Peptide Institute (Osaka, Japan). BCθ, a protease-nicked and biotinylated derivative of θ-toxin, was prepared as described previously [20].

Cells. Human fetal lung fibroblasts, TIG-1, were established from a fetus (5 months old; female) at the Tokyo Metropolitan Institute of Gerontology [27]. Cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin). Cells were passed serially in vitro and used as young and senescent cells at population doubling levels (PDLs) of 22–26 and 61–64, respectively.

Detachment of cells from culture dishes and preparation of detergent-insoluble, low density membrane fractions (raft fractions). Cell homogenates were prepared by the following three methods (see also Results). In the first method (Mechanical detachment method), cell monolayers in 10-cm dishes were washed with phosphate-buffered saline (PBS), and mechanically detached from the dishes with rubber policemen in TNE buffer (25 mM Tris–HCl, pH 6.8, 150 mM NaCl, and 1 mM EGTA) containing 1 mM PMSF, 200 μM leupeptin, 20 μg/ml pepstatin A, 20 μg/ml aprotinin, and phosphatase inhibitor cocktail set II (Calbiochem) (TNE buffer with inhibitor cocktails). The detached cells were collected by centrifugation, incubated with 1% Triton X-100 in TNE buffer with inhibitor cocktails for 15 min on ice, and homogenized with a Potter-Elvehjem homogenizer. In the second method (Detergent pretreatment method), the cells were washed with PBS and then the cells adhering to the dish were first solubilized by incubation with 1% Triton X-100 in TNE buffer containing inhibitor cocktails for 15 min on ice. The cells were then harvested from the dish with a rubber policeman and homogenized as above. In the third method (Trypsinization method), the cells were washed with PBS, and then incubated with 0.25% trypsin for 15 min at 37 °C for cell detachment. The trypsin was inactivated by the addition of culture medium containing 10% FBS, and the cell suspension was transferred to a siliconized tube and washed once with culture medium containing FBS and twice with PBS at 4 °C. The cells were then incubated with 1% Triton X-100 in TNE buffer containing inhibitor cocktails for 15 min on ice and homogenized as above. Cell homogenates prepared by the above three methods were each then mixed with an equal volume of 80% sucrose and overlaid with 35% sucrose and 5% sucrose in TNE buffer. The gradients were centrifuged at 250,000*g* for 18 h at 4 °C in an SW41 rotor. After centrifugation, fractions (total 11 fractions) were collected from the tops of the tubes. The pellet was suspended in TNE buffer, sonicated, and designated as the 12th fraction.

BCθ binding. After washing with PBS, the cells adhering to the dishes were incubated with 10 μg/ml BCθ in PBS containing 1 mg/ml BSA for 30 min on ice and washed twice with PBS. Then the BCθ-bound cells were detached, homogenized, and fractionated either by the Mechanical detachment method or by the Detergent pretreatment method as described above. In some experiments, cell suspensions prepared by the Trypsinization method were incubated with BCθ in siliconized tubes for 30 min on ice, washed, solubilized with Triton X-100, and subjected to density gradient fractionation.

Detection of proteins and lipids. Proteins were analyzed using a bicinchoninic acid (BCA) protein assay kit (Pierce). To analyze the distributions of BCθ, raft-associated proteins, and ganglioside GM1 in the gradient, equal volumes of each fraction of the gradient were loaded onto a gel and SDS–PAGE was performed. The molecules in the gels were transferred to PVDF membranes and visualized using ECL plus (Amersham Bioscience, Piscataway, NJ, USA). The amounts of samples for SDS–PAGE were normalized for total protein content in the gradient (sum of fractions #1–12). Lipids in the cell homogenates and gradient fractions were extracted by the method of Bligh and Dyer [28] with slight modification. Cholesterol was quantified using an assay kit for free cholesterol, Determiner L FC (Kyowa Medex, Japan).

Results

Cellular aging-dependent changes in lipid rafts following mechanical detachment of cells

In analyzing the constituents of lipid rafts in human diploid fibroblasts, TIG-1, we found that cell detachment from the dishes might cause their cellular aging-dependent changes. Lipid rafts were prepared from TIG-1 cells at