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Loss of Functional Caveolae During Senescence of Human Fibroblasts

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Primary human fibroblasts have a finite replicative lifespan in culture that culminates in a unique state of growth arrest, termed senescence that is accompanied by distinct morphological and biochemical alterations. Senescent cell responses to extracellular stimuli are believed to be altered at a point after receptors are bound by ligand, leading to improper integration of the signals which initiate DNA replication. In this study we demonstrate that one of the key organizing membrane microdomains for receptor signaling, caveolae, are absent in senescent cells. A comparison of young and senescent cells indicated that senescent cells contained a higher total amount of caveolins 1 and 2 but had significantly less of both proteins in the caveolar fraction. Additionally, caveolar fractions from senescent cells completely lacked the tyrosine-kinase activity associated with functional caveolae. Furthermore, old cells had little caveolar protein exposed to the outer plasma membrane as estimated by using an in vivo biotinylation assay and no detectable caveolin 1 on the cell surface when processed for immunofluoresence and confocal microscopy. Together, these data suggest that a fundamental loss of signal integration at the plasma membrane of senescent cells is due to the loss of signaling competent caveolae. J. Cell. Physiol. 187:226–235, 2001. © 2001 Wiley-Liss, Inc.

Primary fibroblasts have a finite replicative potential in culture, culminating in the inability of cells to divide even when mitogenic cues are present (Hayflick and Moorhead, 1961; Hayflick, 1965). Senescing fibroblasts progressively acquire a flattened morphology with an increased cellular volume, an irregular shape and show increased secretion of components of the extracellular matrix (ECM) (reviewed in Wheaton et al., 1996). There are many biochemical hallmarks that accompany this phenotype, including the upregulation of $p21^{Waf1/Cip1/Sdi1}$(Noda et al., 1994), p16 (Alcorta et al., 1996; Hara et al., 1996; Wong and Riabowol, 1996), cyclin D_1 (Dulic et al., 1993; Lucibello et al., 1993; Atadja et al., 1995b) and cyclin D_2 (Meyyappan et al., 1998), increased activity of p53 (Atadja et al., 1995a; Vaziri et al., 1997), and Rb (Stein et al., 1990) and a blunted immediate early gene response after serum stimulation (Seshadri and Campisi, 1990; Riabowol, 1992; Atadja et al., 1994; Meyyappan et al., 1999). Although extensive study has focused on telomere shortening as a potential regulator of the development of cellular senescence (Greider, 1998), the events that lead to the manifestation of the senescent phenotype such as blocked growth, morphological differences, and biochemical changes are not well defined. Many of the early studies investigating the loss of mitogenic response of senescent human diploid fibroblasts focused upon growth factor receptor:ligand interactions (Phillips et al., 1983; Gerhard et al., 1991; Sell et al., 1993). In the cases of EGF, Dexamethasome, and IGF-1 it was found that the number of receptors remain the same per unit surface area in senescent cells and that the ligand affinity remained unchanged (Sell et al., 1993). Similarly the binding kinetics of the glucocorticoid receptor, and the insulin receptor have been shown to remain unaltered in senescent cells (Chua et al., 1986). It is, however, established that the downstream responses of growth factors, such as the induction of the immediate early genes c-fos and Egr-1 are blunted in senescent cells (Seshadri and Campisi, 1990; Riabowol et al., 1992; Meyyappan et al., 1999). Thus, the emerging consensus is that the senescence specific growth block is not due to alterations in growth factor-receptor engagement or processing, but is due to a postreceptor block which develops and leads to reduced immediate early gene responsiveness. Several studies have attempted to identify the points at which signaling cascades may be modified in senescent cells. For example, an age dependent decline in mitogenic

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stimulation of rat hepatocytes has been reported to be caused by a reduced association between Shc and the epidermal growth factor receptor (Palmer et al., 1999; Hutter et al., 2000). Conversely, the activation of the Ras/Raf/MAPK pathway in primary fibroblasts has been shown to lead to aspects of senescence (Lin et al., 1998; Zhu et al., 1998). Another study demonstrated that the PI3K pathway, when inhibited by LY294002, can also lead to senescent cell characteristics (Tresini et al., 1998), but this occurred when using concentrations of drug that may be sufficient to inhibit other PI3K family members, DNA-PK and ATM (Rosenzweig et al., 1997; Hosoi et al., 1998; Sarkaria et al., 1998). Thus, although the general consensus is that receptor-ligand interactions are maintained in senescing cells, the basis for the alterations in components of the downstream pathway itself are presently unknown. In order to address this question a membrane microdomain structure which is believed to be crucial to mitogenic signal organization, caveolae, was examined in senescent and proliferative primary fibroblasts.

Caveolae are flask shaped invaginatons which form at the plasma membrane from lateral assemblies of cholesterol and sphingolipids known as rafts (reviewed in Harder and Simons, 1997). Caveolae and other lipid rafts are characterized by their unique lipid composition, and can be biochemicaly purified based on their insolubility in the detergent Triton X-100 as well as their low buoyant density on sucrose gradients (reviewed in Anderson, 1998). The catalyst of caveolar formation is the oligomerization of a class of sphingolipid and cholesterol binding proteins known as caveolins (Monier et al., 1996), which have been suggested to stabilize and induce the lipid rafts to coalesce (reviewed in Harder and Simons, 1997). Three caveolin genes have been identified, encoding caveolin proteins 1, 2, and 3 (Glenney, 1992; Kurzchalia et al., 1992; Scherer et al., 1996; Tang et al., 1996; Way and Parton, 1996). Caveolin monomers oligomerize into hetero- and homo-dimers and trimers as they are translated in the cytoplasm (Lisanti et al., 1993), traffic through the trans-golgi network and associate at the plasma membrane (reviewed in Rietveld and Simons, 1998). Mounting evidence suggests that many well-studied receptor signaling systems may be localized to and operate through the caveolae. These include Src family kinases, nitric oxide synthase, EGFR, PDGFR, PLCγ, PLD, PKCα & PKCβ, Ras, Trimeric G-proteins, MEK, and Erk2, among others (reviewed in Kurzchalia and Parton, 1999). Thus, the caveolae may act as a center of cross talk and integration of mitogenic responses in normal cycling cells. More importantly, evidence suggests that many of the pathways shown to be altered in, or which may directly influence senescence are localized to the caveolae such as the MAPK, PI3K, and PLD pathways (Lin et al., 1998; Tresini et al., 1998; Zhu et al., 1998; Venable and Obeid, 1999). Considering that a profound post receptor block of mitogenic signaling is associated with the development of senescence, the microdomains where receptors and signaling cascades are believed to be linked required investigation. We demonstrate that primary senescent fibroblasts have a significant reduction in signaling competent caveolae based upon membrane localization and functional characterization. The potential reasons and consequences for this major morphological alteration are discussed.

MATERIALS AND METHODS

Culturing of primary human fibroblasts

Human Hs68 fibroblasts (American Culture Collection CRL no. 1635, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1mM glutamine and 10% (vol/vol) fetal bovine serum. It has been previously established that under conditions we use, this cell line undergoes replicative senescence at a mean population doubling ranging from 80 to 86, with a [^{3}H] thymidine incorporation of less than 10% (typically 3–5%) of the cell population within 36 h. Senescent cells chosen for this study had undergone 81 MPDs, did not reach confluence two weeks after subculturing, demonstrated a characteristic flattened senescent morphology and were positive for senescence associated β-galactosidase (β-gal) activity. Young fibroblasts used in the study were at an MPD of 42, doubled approximately every 24 h, and did not stain blue in the acidic β-gal assay. Before harvesting or treatment with mitogens the cells were serum starved for 48 h. A subgroup of both young and old cells were pretreated with a 10 ng/ml EGF bolus, 10 min before harvest.

Fractionation and purification of caveolar enriched fractions by sucrose gradient centrifugation

To evaluate the caveolae content within particular cells, caveolae fractions were prepared as described previously (Robbins et al., 1995). Briefly, fibroblasts were harvested with a 1% Triton X-100 lysis buffer and protein samples were quantified (BCA protein assay kit, Pierce) prior to homogenization and sucrose gradient centrifugation. A 40%, 30%, or 5% sucrose step gradient and analytical centrifugation were used to isolate the flocculent, dense white band enriched in caveolae which appeared at the interface between the 30 and 5% sucrose layers. This triton insoluble layer was isolated by centrifugation and resuspended in 2 $\times$ SDS sample buffer (for caveolae samples), biotin solution (for biotin labeling), or 50 mM Tris buffer (for cold kinase assays). In addition to the isolation of caveolar fractions from the sucrose gradient, samples enriched in cytoplasmic proteins were isolated from the lower portion of the gradient for use as controls. These were similarly resuspended in 2X SDS sample buffer for western analysis.

Western blotting and kinase assays

Loading gels for the Western blot analyses were run to ensure that the fractionation procedure isolated comparable amounts of protein from both young and old cells. Due to the wide range of protein concentrations in different fractions, silver staining was used to quantify fractionated samples while Coomassie brilliant blue was used to stain total extracts. The caveolar and cytoplasmic fractions described above were electrophoresed through 10% polyacrylamide gels and transferred onto nitrocellulose for 1.5 h at 70 V in a transfer buffer containing 120 mM Tris Base, 40 mM glycine, and 20% methanol. Antibodies were diluted and membranes

were blocked overnight in TBS containing 5% nonfat milk, 0.1% Tween-20, and 0.1% NP-40. Antibodies for flotillin (Transduction Laboratories F65020), caveolin 2 (Transduction Laboratories C57820), caveolin 1 (Transduction Laboratories C13620), and Fyn (Transduction Laboratories F19720) were prepared at recommended dilutions and incubated with membranes for 1 h at room temperature. The secondary sheep anti-mouse biotinylated antibody (Amersham RPN1001), and the tertiary antibody strepavidin-horse radish peroxidase (SA-HRP: Amersham RPN1231) were used at 1/1000 and incubated with membranes for 1 h at room temperature.

Kinase assays on purified caveolar fractions were performed in vitro to assess the kinase activity in fractions from senescent and young cells. Caveolar fractions were incubated in the presence of 1 mM ATP, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ in kinase buffer and incubated for 10 min at room temperature. Reactions were stopped by adding 2X SDS sample buffer and the samples were then resolved on 10% SDS-PAGE gels.

Biotin labeling

Purified caveolar fractions were labeled with biotin in vitro by exposing the fractions to 13 ng/ml Sulfo-NHS-LC-biotin (Pierce, Rockforty, IL) and incubating at room temperature for 10 min. The samples were then mixed with 2X SDS sample buffer and loaded onto 10% SDS-PAGE gels, transferred, blocked (as described above) and probed with Strepavidin HRP (Amersham). Labeling of intact cells was carried out by exposing a fibroblast monolayer to Sulfo-NHS-LC-biotin in PBS for 10 min which were washed thoroughly before harvesting and caveolar fractionation (as described above). The biotinylated caveolar fractions were electrophoresed (10% PAGE), transferred and blotted with SA-HRP as described above.

Immunofluorescence and confocal microscopy

Hs68s were fixed for 20 min in 100% methanol at $-20^{\circ}C$, washed in phosphate buffered saline (PBS), and incubated for 1 h at room temperature in PBS containing 1% bovine serum albumin and caveolin-1 monoclonal antibody (Transduction Laboratories C13620). After washing in PBS, cells were incubated for 1 h at room temperature in secondary antibody diluted 1:500 (goat anti-mouse conjugated with Cy3; Jackson Immunoresearch), washed in PBS and observed by fluorescence microscopy. Digital deconvolution was performed using a 14 bit cooled CCD camera (Princeton Instruments) mounted on a Leica DMRE immunofluorescence microscope. VayTek microtome digital deconvolution software was used to remove out of focus signal, and image stacks were projected into one image plane using Scion Image software. Coloration and superimposition of images was performed with Adobe Photoshop 5.0.

RESULTS

Sucrose gradient fractionation of a triton insoluble fraction in both young and senescent cells enriches for caveolae/lipid raft microdomains

Differences in lipid and membrane properties between senescent and young cells may influence the buoyant density of these fractions with age (Schroeder et al., 1984; Rutter et al., 1996). Therefore, it was necessary to test whether comparable amounts of protein could be isolated from both young and old caveolar-enriched fractions. Electrophoresis and silver staining of cytoplasmic fractions (Fig. 1A, lanes 3 and 4) showed that comparable amounts of protein were recovered, indicating that similar amounts of protein were loaded on to gradients before fractionation. To further test that the sucrose gradient isolation procedure was effective for both young and old cells, a known marker of lipid rafts, flottillin (Bickel et al., 1997), was visualized in a western blot of the same fractions. Flotillin was clearly detected in both young and senescent fractions (Fig. 1B, lanes 1 and 2) and in total extracts (Fig. 1B, lanes 5 and 6), but not in the cytoplasmic fraction (Fig. 1B, lanes 3 and 4). Thus, the detergent extraction procedure enriched for potential caveolar containing domains in both young and old cells, although the total amount of protein in the fraction was somewhat reduced in old cells (Fig. 1A, lanes 1 and 2). Consistent with this, slightly less flottillin was seen in the senescent cell caveolar preparation, despite a modest increase in total cell extracts (Fig. 1B). It should be noted that the caveolar fractionation procedure isolates both the components of the caveolar structures and the lipid rafts which form them. Currently there is no known way of efficiently isolating these lipid populations from each other, and so the triton insoluble fractions used in this study are homogenous for the components of both caveolae and lipid rafts. Thus, these fractions are most accurately described as caveolar enriched, but cannot be defined as originating from caveolae unless they are caveolin positive.

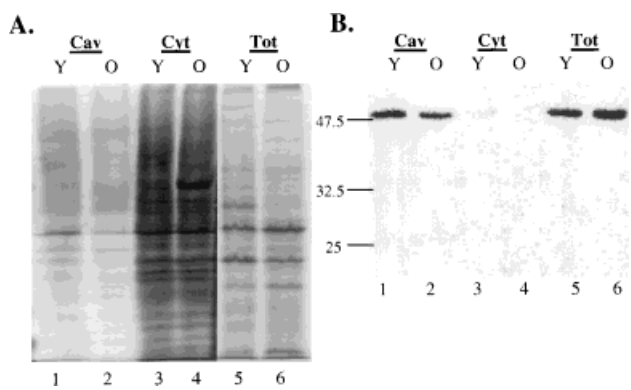

Fig. 1. Sucrose density gradient fractionation isolates a triton insoluble protein containing fraction from both young and old cells which have a marker for the caveolar fraction. **A:** Silver stain and Coomassie stain loading gels. Both young (Y) and old cells (O) were fractionated as described in materials and methods to caveolar rich (Cav), and cytoplasmic (Cyt) fractions by sucrose density separation of the Triton-insoluble extract. Additionally total cellular lysate (Tot) was collected from young and old fibroblasts. These fractions were run on a 10% SDS PAGE, and silver stained in the case of fractionation or coomassie stained in the case of total extracts to quantify protein loading for westerns. Additionally these gels indicate the relative protein amounts of the caveolar fraction. **B:** Flotillin western blot of caveolar enriched fractions. Fractions were isolated as described above and were blotted to nitrocellulose. A western blot using anti-flotillin was performed on these samples. Flottillin acts as a marker of the caveolar fraction, and indicates that fractions with similar properties and cellular location were isolated from both young and old cells.

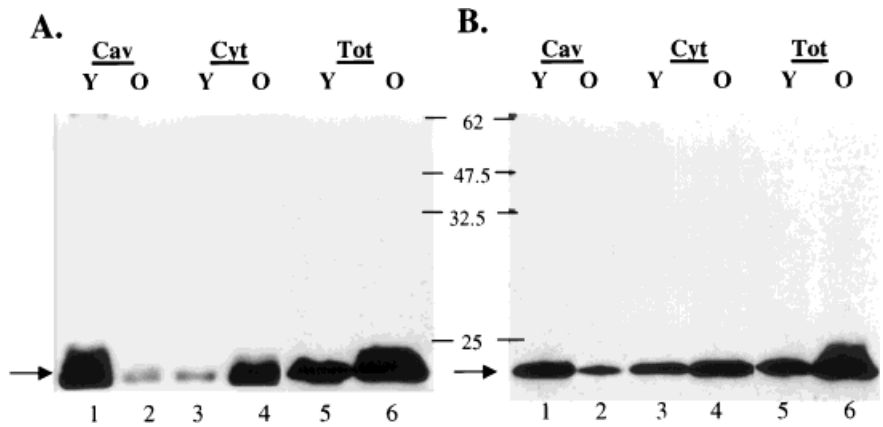

Fig. 2. The Senescent fibroblast caveolar fraction contains a lower amount of caveolin protein. Both Young (Y), MPD = 40, and Old (O), MPD = 81 Hs68 fibroblasts were serum starved 24 h prior to fractionation on a sucrose gradient as described in materials and methods to obtain cytoplasmic (Cyt) and caveolar enriched (Cav) fractions. Additionally, young and old cells were harvested directly into sample buffer to obtain a total (Tot) cell extract. These fractions were western blotted with caveolin antibodies. **A:** Blot of fractions with caveolin 1 antisera. **B:** Blot of fractions with caveolin 2 antisera.

Caveolar enriched fractions of senescent cells have decreased amounts of caveolin 1 and caveolin 2

Caveolar enriched and cytoplasmic fractions from both young and old fibroblasts were electrophoresed and western blotted to detect caveolin 1 and caveolin 2 (Fig. 2). Both caveolin 1 (panel A) and 2 (panel B) protein were reduced in the caveolar enriched fraction isolated from old cells (Fig. 2A and B, lanes 1 vs. 2), despite the fractions from old cells containing significant levels of flotillin (Fig. 1B). Conversely, the caveolin 1 and 2 protein levels seen in the cytoplasmic fraction were higher in old cells compared to young (Fig. 2A and B, lanes 3 vs. 4). Increased caveolin 1 and 2 are also seen in the total extract of senescent fibroblasts (Fig. 2A and B, lanes 5 vs. 6). Thus, it appears that the caveolin 1 and 2 proteins are produced at high levels in old cells but do not localize to the detergent insoluble caveolar fraction.

Caveolar enriched fractions from old cells lack tyrosine kinase activity

To test whether caveolae from old cells were signaling competent, equal amounts of caveolar enriched and cytoplasmic fractions from both young and old fibroblasts were electrophoresed and blotted with a phosphotyrosine antibody (4G10). As seen in Figure 3A, two peptides heavily phosphorylated on tyrosine residues are detected in young cells, compared to a barely distinguishable signal in senescent cell caveolar enriched fractions. The phospho-peptides in this molecular weight range of the caveolar enriched fraction likely represent Src family kinases, since members of this family are known to be localized to, and phosphorylated in, fibroblast caveolae (Shenoy-Scaria et al., 1994; Robbins et al., 1995; Mastick and Saltiel, 1997). This assumption is consistent with a Fyn western blot (Fig. 3C, lanes 1 and 2), which demonstrates that the Src family kinase Fyn is present in the caveolar enriched domains of young, but not old fibroblasts. Interestingly, phospho-peptides of similar size are seen in the cytoplasmic fraction of both senescent and young fibroblasts (Fig. 3A, lanes 6 and 8). This suggests that the same peptides are phosphorylated in both young and old fibroblasts, but the phosphorylated forms do not localize to caveolae in senescent cells.

To further investigate the signaling competence of caveolae with cell passage, caveolar enriched fractions from young and old cells were used in kinase assays (Fig. 3B). Native kinase activity of the fractions was assessed by western blot with the 4G10 antibody. The caveolar enriched fraction from old cells had no detectable in vitro kinase activity when compared to the clear activity seen in young cells (Fig. 3B, lanes 1 vs. 3). Lanes 2 and 4 of Figure 3B show a control assay without ATP, demonstrating that the phosphorylation events observed occurred de novo, and were not due to kinase activity prior to isolation. These results show that functionally intact caveolae cannot be isolated from senescent cells, whereas the same fractions from young cells clearly have strong in vitro activity.

In order to begin to identify which pathways may be altered in senescent cell caveolar fractions, a Src family kinase which is often found associated with caveolae (reviewed in Resh, 1998), Fyn, was probed for in the caveolar enriched fractions of both young and old cells (Fig. 3C, lanes 1 and 2). Fyn was detected in the young caveolar enriched fraction, but not in the senescent cell fraction even though a significant amount of Fyn is detectable in the total lysates of both young and old fibroblasts (Fig. 3C, lanes 9–12).

Epidermal growth factor stimulation leads to caveolar localization, but not to phosphorylation of Fyn

The downstream events of EGFR signaling were chosen as possible sites of differential regulation in senescent cells due to the reported altered interaction with Shc during aging in hepatocytes (Palmer et al., 1999; Hutter et al., 2000), and the previous detection of this receptor in caveolae by several groups. An EGF bolus was delivered to both young and senescent cells, but it had no detectable effect on signal cascades in the caveolar enriched fraction (Fig. 3A and B), or on caveolin distribution (data not shown). Thus, although EGF

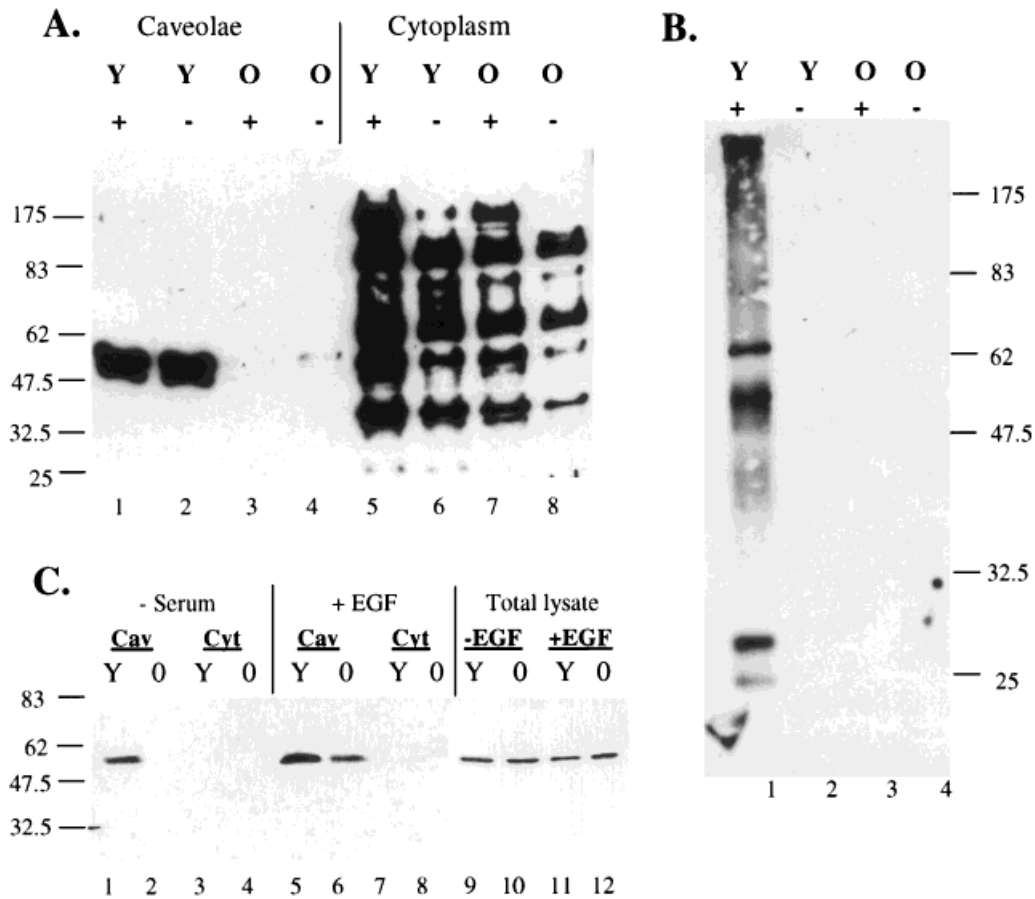

Fig. 3. Senescent fibroblasts do not have functional caveolae. **A:** Phospho-tyrosine blot of caveolar fractions. Both young (Y) and old (O) Hs68 fibroblasts were serum starved for 24 h and stimulated with EGF(+) or left unstimulated (−) as described in the materials and methods. Young (MPD = 40) and Old (MPD = 80) Hs68 fibroblasts were fractionated on a sucrose gradient as described in materials and methods to obtain cytoplasmic and caveolar enriched fractions. These fractions were blotted with 4G10 antisera, which is specific for phosphorylated tyrosine residues. **B:** Phospho-tyrosine blot of caveolar kinase assay. Caveolar fractions in this case was resuspended in kinase buffer and a kinase assay performed as described in materials and methods. Products of the caveolar kinase assay were visualized with a western blot using a 4G10 antibody as described above. **C:** Anti-Fyn blot of caveolar fractions. Fibroblast culturing and fractionation were performed as previously described for both young (Y) and Old (O) Hs68 fibroblasts. The cytoplasmic (Cyt) and caveolar (Cav) fractions were blotted with a monoclonal human Fyn antibody. Additionally the total lysate from young and old cells, either serum starved (− EGF) or EGF stimulated (+ EGF) was blotted with the Fyn antibody. Comparison of band intensities using densitometry determined there was a 2.75 ± 0.04-fold increase between lanes 5 and 6.

appeared to initiate signal cascades in both young and old cells it had little effect on the signal generated within the caveolar fraction (Fig. 3A, lanes 1–4). It would appear that the protocol used to isolate the caveolar fractions does not allow active EGFR or any of its downstream phospho-peptides to co-purify. This was confirmed with an EGF-receptor western blot, which could not detect the protein in the caveolar fractions of young or old cells, but could in the cytoplasmic fractions of young and old cells (data not shown). This was expected since isolation of the EGFR with caveolae is only possible using a non-detergent method of fractionation (Anderson, 1998). However, EGF stimulation does lead to a 2.75 ± 0.04-fold increase (as determined by densitometry) in localization of Fyn to the caveolar-enriched microdomain in young compared to senescent cells (Figs. 3C, lanes 5 and 6). This EGF stimulation does not alter caveolin protein levels (data not shown), implying that Fyn is recruited to existing microdomains. Thus, it appears that although Fyn is recruited to the caveolar fraction after EGF stimulation, this new kinase population is not phosphorylated in either the senescent or young cell caveolar enriched-fractions.

Caveolin is not localized on the membrane surface of senescent cells

Although the markers and functional parameters of caveolae were not detectable in senescent cell caveolar-enriched fractions, this difference may be a result of the unique lipid composition of senescent cells which could prevent isolation of intact caveolar domains. In order to address this issue it was necessary to determine whether identifiable caveolar structures could be detected in the plasma membrane of young and senescent fibroblasts by caveolin 1 immunoflouresence.

Figure 4 shows that high concentrations of caveolin 1 are found on the outside periphery of young cells, proximal to the plasma membrane as indicated by the

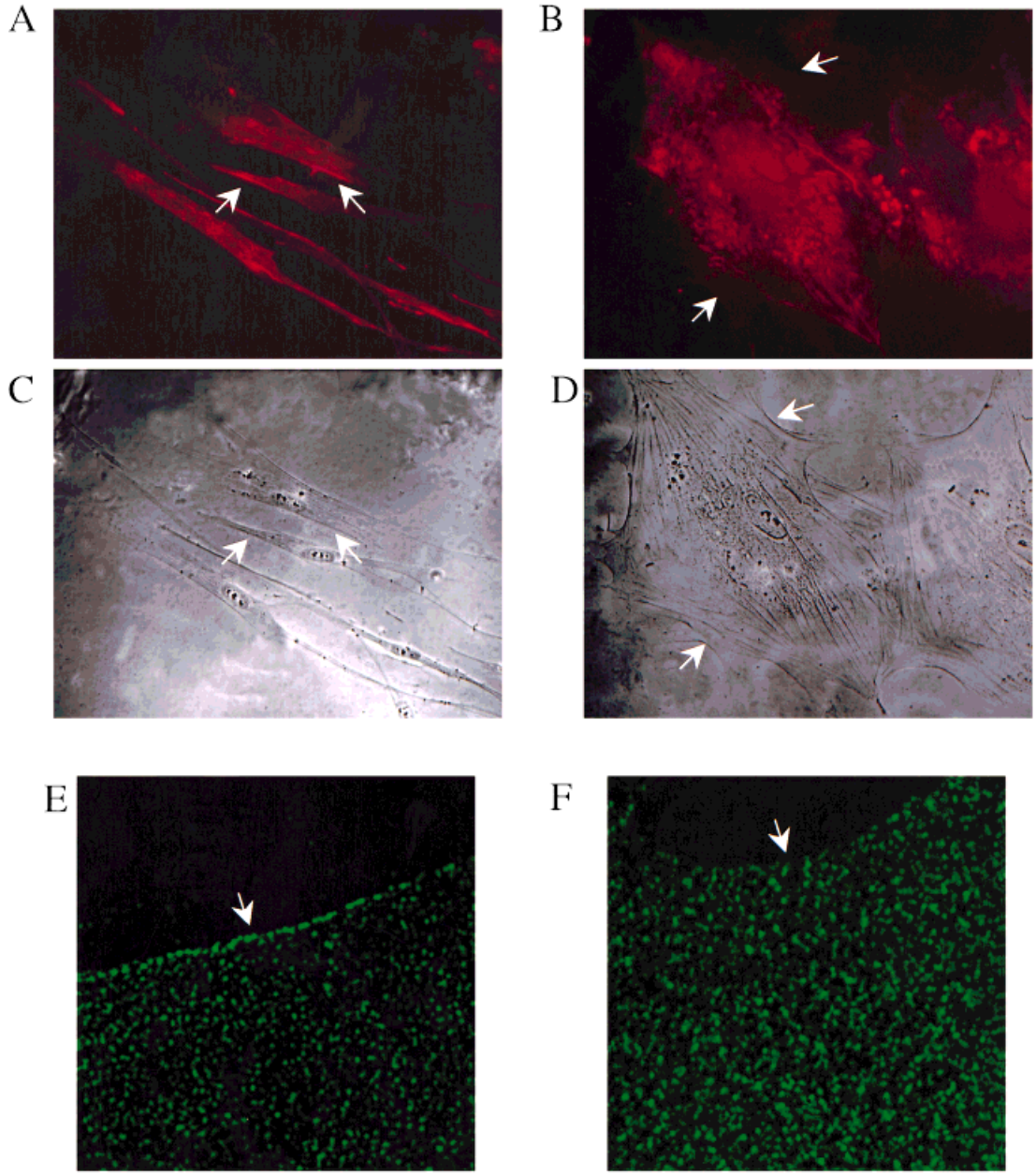

Fig. 4. Caveolar markers do not physically localize to the plasma membrane of senescent fibroblasts. **A-D:** Immunofluorescence using caveolin 1 rabbit antisera and a Cy3 secondary was performed on fixed and triton treated Young (A) and Senescent (B) Hs68s. A differential interference contrast (DIC) image from the same field was also captured for both young (C) and senescent Hs68s (D). White arrows point to membrane sections of young and senescent cells, to allow comparison between fluorescence and DIC images. **E** and **F:** Confocal immunofluorescence using caveolin 1 antisera was used to detect caveolae structures of fixed and Triton-solubilized fibroblasts. These images focus on the leading edge of the cell body at 250× magnification. The images show the results of deconvolution microscopy as described in methods and materials for both senescent and young Hs68 fibroblasts. White arrows point out the membrane edge of both young (E) and senescent (F) cells.

arrows in panels A and C. In contrast, caveolin shows a diffuse cytoplasmic staining pattern in senescent cells (Fig. 4B and D), consistent with results obtained by biochemical fractionation. Thus, there is a distinct difference in localization between caveolin 1 in senescent and young cells, suggesting that caveolar structures are lost from the plasma membrane as cells age. Young and old cells stained with caveolin 1 were also examined by confocal microscopy. Confocal microscopy images were focused on one edge of the cell body at high magnification to determine caveolin distribution between the membrane and cytoplasm. We reproducibly observed a strong membrane signal in young cells, but only a diffuse cytoplasmic signal in old cells. (Fig. 4, panels E vs. F). Thus, immunolocalization of caveolin 1 corroborates biochemical data, implying that caveolin

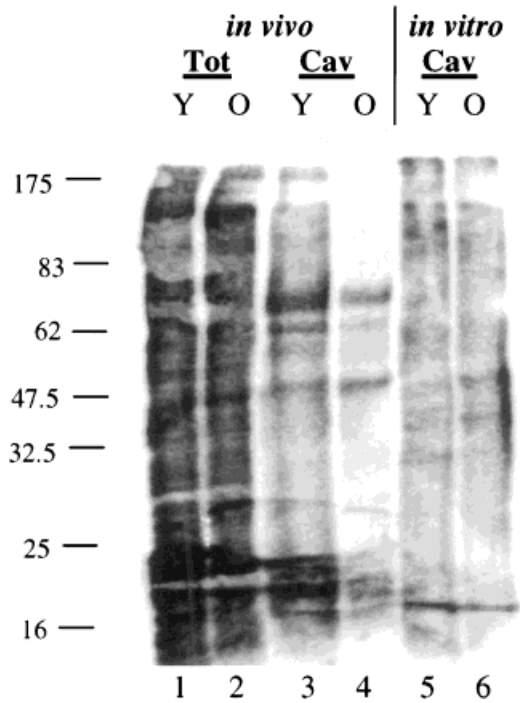

Fig. 5. Caveolar -specific biotin labeling. Intact Hs68 cells were exposed to NHS-LC-biotin and harvested whole to obtain a total (Tot.) in vitro fraction, or were separated into caveolar enriched (Cav) fractions as described in the materials and methods. The in vitro biotin reaction was carried out on a previously isolated caveolar enriched (Cav) fraction from young and old cells. All reactive proteins exposed to the outside of the cell or membrane fraction are visualized with SA-HRP on a western blot.

does not localize to the membrane and consequently caveolae do not form in senescent cells. Immunofluoresence data further supports this possibility since more total caveolin signal is seen in the cytoplasm of senescent cells (Fig. 4, panels E vs. F), which is also reflected in the caveolin 1 western blot of senescent cell cytoplasm (Fig. 2A, lanes 6 vs. 8).

If caveolae are reduced in number at the surface of senescent cells, then proteins within these structures should not be exposed to chemical modification in intact cells. Thus, to examine caveolar structures in old cells by a direct approach, biotinylation was performed on intact young and senescent cells. Cells were than fractionated as previously described into caveolin rich membrane fractions. A western blot of these caveolar fractions with strepavidin conjugated to horseradish peroxidase revealed peptides which were exposed to the outer leaflet of the plasma membrane in intact cells. Figure 5 shows that biotinylation of proteins is reduced in the caveolar enriched fraction of old cells compared to that of young (lanes 3 vs. 4), but in vivo biotinylation of intact cells before fractionation shows similar amounts and species of biotinylated proteins (lanes 1 and 2). Another control using caveolar fractions showed that the amount of protein in the caveolar fractions labeled in vitro is similar in young and old fractions (Fig. 5, lanes 5 and 6). Thus differential labeling of proteins with biotin is only achieved when intact cells are exposed to the biotin reaction in vivo. These results demonstrate that only low amounts of caveolae and lipid raft-associated proteins are available for biotinylation on the senescent cell surface.

DISCUSSION

This study establishes for the first time that many of the characteristics of functional caveolae are absent in senescent fibroblasts. These include lack of caveolin localization to the caveolar enriched fraction, no evidence of kinase activity in the caveolar enriched fraction and absence of membrane immunolocalization of caveolin. It has been shown in a variety of studies that caveolins oligomerize to form caveolar structures (Lisanti et al., 1993; Monier et al., 1995; Monier et al., 1996; Scherer et al., 1997) and that overexpression of caveolin 1α can drive the formation of caveolae structures (Fra et al., 1995; Engelman et al., 1997). Furthermore the presence of both caveolin 1 and 2 have been shown to be pivotal in the proper formation and stability of caveolar structures (Engelman et al., 1997; Scherer et al., 1997; Li et al., 1998; Okamoto et al., 1998). Thus, the lower amount of both caveolin 1 and 2 in the caveolar enriched fraction of senescent cells suggests that these microdomains do not form normally in old cells. Additionally, the caveolin 1 and 2 proteins are more abundant in the cytoplasmic fraction of senescent cells, implying that localization to rafts or caveolae is inhibited.

Caveolin 1 could not be detected in the plasma membrane of intact senescent cells by either direct immunolocalization or immunoconfocal microscopy, but is clearly localized to the membrane in young cells. Additionally, biotinylation of intact cells is able to label proteins in the caveolar enriched fraction much more intensely in young cells. Together, these two localization experiments suggest that few, if any, caveolae are present in the membrane of senescent cells. Collectively, the above data suggest a model in which senescent cells are unable to organize and localize the components of functional caveolae to the plasma membrane. Although the expression of caveolin has been shown to be sufficient to drive caveolae formation, other factors are clearly necessary since senescent cells had few caveolae, even in the presence of high levels of caveolin 1 and 2 expression.

The observations above could be explained by previous reports which describe the unique properties and composition of the plasma membrane of old cells (Schroeder et al., 1984; Rutter et al., 1996). Rafts are comprised of a high concentration of sphingolipids and cholesterol, which have strong cohesive forces that counteract the entropic force inherent in a fluid mosaic membrane (reviewed in Harder and Simons, 1997). In senescent cells it has been shown using proton magnetic resonance (PNR) that the ratio of cholesterol/phosphatidylcholine increases as MRC-5 fibroblasts age in culture, indicating an increased amount of mobile cholesterol (Rutter et al., 1996). Another report sought to probe the lipid composition of these fibroblasts by using fluorescence probe molecules, which could be used to determine the limiting anisotropy (fluidity) of membranes, and showed that the microsomal fraction increased in fluidity with donor age (Schroeder et al.,

1984). Considering that lipid composition changes during fibroblast aging lead to higher lipid fluidity it may reflect the inability of significant raft domains to form with age, since rafts are islands of less fluid lipid. Alteration of the raft composition or dynamics may interfere with raft coalescence or its ability to sequester caveolin proteins. The increase in caveolin 1 and 2 in the cytoplasm of old cells supports this model, since it would indicate that the proteins are unable to associate with rafts due to altered dynamics or composition, and subsequently they are not transported to the cell surface.

The ability of caveolae to act as sites of signal transduction also appeared to be impaired in the caveolar enriched fractions of senescent cells. The 4G10 phospho-tyrosine antibody revealed no detectable phospho-peptides in the caveolar fractions of senescent cells, compared to an abundant signal in young cells. Additionally, the in vitro kinase assays suggested that the senescent cell caveolar fractions are not signaling competent in comparison to young cells. Furthermore, the Fyn kinase did not localize to the caveolar fraction of serum starved old cells, and was only modestly induced to translocate to caveolae in response to EGF. Considering that Fyn is associated with fibroblast anchorage dependent growth (Wary et al., 1996, 1998) and that blocking these Src kinases leads to G-phase block in NIH 3T3 cells (Roche et al., 1995), it is likely that this kinase plays a significant role in EGF and PDGF mitogenic signaling. The reduction of Fyn localization and lack of phosphorylation in senescent cell caveolar enriched fractions suggest that these receptors may not be able to efficiently couple with Fyn in response to ligand. Together, these data indicate that the signaling-competent caveolar fraction is significantly reduced in senescent cells. It, therefore, appears that the intact caveolar kinase cascade which can be visualized in young cells is missing essential components in old cells or is improperly integrated in the caveolar enriched fraction.

The functional implications of the absence of caveolae in senescent cells are many fold. Key cellular pathways that operate through these structures which are activated or misregulated could lead to aspects of the senescent phenotype. Considering the great number of kinase cascades that have been localized to caveolae, it is tempting to predict that one or more of these may modulate the mitogenic response in primary fibroblasts. Indeed, the absence of caveolae may prevent mitogenic signal propagation at all, considering that many growth receptors have been found to be localized within, and to signal through caveolar structures. Thus, the post receptor block could be the result of receptors being uncoupled from caveolae or rafts that are subsequently unable to maintain integrated downstream responses to ligand. Indeed, one example of this is EGF signaling in senescent cells where altered EGFR proteolytic processing (Carlin et al., 1994), altered Shc-EGFR interactions (Palmer et al., 1999; Hutter et al., 2000), altered nuclear translocation of ERK (Kim et al., 2000; Lim et al., 2000), and loss of SRF binding to downstream promoters (Atadja et al., 1994; Meyyappan et al., 1999) have been noted. With the possible exception of proteolytic processing of the EGF receptor, all of these effects appear to be downstream of an altered signal integration or compartmentalization within the plasma membrane caveolar microdomains.

Although the literature suggests that the EGF response is integrated within caveolae, our data show that EGF receptor engagement leads to Fyn recruitment to caveolar enriched microdomains, but does not lead to Fyn phosphorylation. This result is consistent with previous observations, which show that ephrin leads to recruitment of Fyn to caveolae but does not lead to higher Fyn phosphorylation (Davy et al., 1999). A possible explanation for this observation is that there may be a constant amount of Fyn phosphorylation before and after EGF stimulation, and the newly recruited Fyn is only tyrosine phosphorylated by signals generated from another stimulus. Alternatively, the caveolar structure may be recycled after EGF stimulation, leaving a steady state of phosphorylated Fyn available in the membrane microdomain. Lastly, Fyn may be recruited to lipid rafts which have not coalesced into caveolae in the time frame observed, and thus remain unphosphorylated but still purify with the caveolar fraction. Interestingly, Fyn does not strongly localize to senescent cell caveolae in response to EGF. This reduced Fyn recruitment is proportional to the lack of caveolar markers in the membranes of old cells. Thus the absence of caveolae in senescent membranes is likely why little Fyn is seen in the caveolar fraction. This observation is supported by data from T-cells isolated from elderly patients which show decreased Fyn activity (Whisler et al., 1997, 1999). Although lymphoid cells do not have identifiable caveolar structures they do have flotillin positive raft domains (Robbins, unpublished), which are known to be intrinsic to TCR signaling and which sequester Fyn (reviewed in Simons and Toome, 2000).

During the course of this work a report was published suggesting that increased levels of caveolin 1 in senescent cells leads to a block of EGFR in senescent fibroblasts and that the number of caveolar-like structures (based upon transmission electron microscopy) increase in senescent cells (Park et al., 2000). Our data corroborate their observation that levels of caveolin 1 and 2 proteins increase in senescent cells. However, we were unable to determine any conditions under which bona fide caveolar structures which contain caveolin proteins increase in old cells. Indeed, our data indicate that caveolin proteins do not localize properly to the caveolar fraction and do not immunolocalize to the membrane of senescent cells. The data presented here therefore support the idea that there is a lack of organization or formation of classically defined caveolae during senescence.

The data in this study strongly support the idea that signaling competent caveolae do not exist in the membranes of replicatively senescent cells. Additionally, the absence or reduction of components of mitogenic signaling cascades from senescent cell caveolar fractions, such as Fyn, is consistent with a causal role in the blunted growth response seen during cellular senescence. Collectively, these observations suggest that localization and integration of signaling cascades in caveolae is disrupted in senescent cells. Thus signal transduction pathways that rely upon caveolae for signal integration and propagation, would be unorga-

nized in senescent cells and explain the reduced or absent response to mitogens.

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