Are Cross-bridging Structures Involved in the Bundle Formation of Intermediate Filaments and the Decrease in Locomotion That Accompany Cell Aging?

EUGENIA WANG
The Rockefeller University, New York, New York 10021

ABSTRACT Five different fibroblast strains derived from donors of a wide range of ages were used for investigation of senescence-associated changes in the organization of intermediate filaments (IFs) and the activity of cell locomotion. Results of immunofluorescence microscopy demonstrate that, in large and flat in vitro aged fibroblasts, vimentin-containing IFs are distributed as unusually organized large bundles. Electron microscopic examination shows that these large bundles are indeed composed of filaments of 8–10 nm. Such a profile of large bundles is rarely seen in young fibroblasts whose IFs are usually interdispersed among microtubules. Within the large filament bundles of senescent fibroblasts, cross-bridge-like extensions are frequently observed along the individual IFs. Immunogold labeling with antibody to one of the cross-bridging proteins, p50, further illustrates the abundance of interfilament links within the IF bundles. The senescence-related increase in interfilament association was also supported by the results of co-precipitation between vimentin and an associated protein of 50,000 D. Time-lapse cinematographic studies of cell locomotion reveal that accompanying aging, fibroblasts have a significantly reduced ability to translocate across a solid substratum. These results led me to suggest that the increased interfilament links via cross-bridges may in part contribute to the mechanism that orchestrates the formation of large filament bundles. The presence of enormous bundles in the cytoplasm may physically impede the efficiency of locomotion for these nongrowing cells.

The finding that replication of fibroblasts in culture is restricted to a defined time span introduces the establishment of a methodology for in vitro observation of cellular senescence. Not only have the initial reports of Swim and Parker (26) and Hayflick and Moorehead (8) been repeatedly confirmed in fibroblasts different from the original WI-38, but also this may be observed in cells of nonmesenchymal origin, such as endothelial and smooth muscle cells (see review in reference 20). In contrast to Hayflick's interpretation that loss of proliferative potential by human diploid fibroblasts in culture is a manifestation of aging, Martin et al. (15) and Bell et al. (1) suggest that the cessation of replication can be interpreted as the final event in differentiation.

An increase in cell size and the presence of large rigid filamentous structures are two of the frequently observed phenotypic properties associated with the process of fibroblast senescence (3, 28, 33). Our recent results demonstrate that the filamentous structures commonly seen in the large, nonproliferating senescent cells are composed of numerous actin-containing microfilaments, intermediate filaments (IFs), and an elaborate network of microtubules (29). These cytoskeletal components form a complex three-dimensional structure that occupies most of the cytoplasm.

In apparently normal fibroblasts of a proliferating monolayer culture, individual IFs are in general interdispersed among the microtubules as part of the architectural scaffold of the cytoplasm. Often these fibrous organelles are enmeshed in the finer microtubular filaments (34). These distinct network distribution of IFs can be readily altered into an aggregated filamentous cap adjacent to the nucleus by treatment with colchicine, vinblastine, podophyllotoxin, vanadate, heat shock, or virus infection (2).

I report here that nonproliferating senescent fibroblasts...
contain vimentin-containing fibers as large aggregated bundles swirling randomly in the cytoplasm. Ultrastructural examination reveals the bundles as filaments with a 10-nm diameter linearly aligned. Structurally distinct cross-linking is observed in abundance, extending perpendicularly between individual filaments to form interfilament links. The increase in the interfilament relationship is further supported by the co-precipitation of vimentin and a protein of 50,000 D found in cell extracts derived from senescent fibroblasts, but not in their young counterparts. Parallel to these changes in the IF system, reduced activity of cell locomotion is also observed to accompany in vitro aging. The significance of these observations that relate to the physiological function of fibroblasts will be discussed.

**MATERIALS AND METHODS**

**Cell Culture:** The cells used were five selected cell strains derived from donors covering a wide age range from 8 fetal wk to 66 yr (Table I). These five strains were originally purchased from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Institute for Medical Research (Camden, NJ). The cell strains received in the form of cultures in T-75 flasks were cultured initially in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 1% nonessential amino acid. As soon as the cell cultures became confluent, a strict schedule of subcultivation was followed. The in vitro life history of each cell strain was defined by the recording of the number of population doublings per passage and the time involved, and the cumulative number of actual population doublings (CPDL) during passage to the end of the proliferative life span of a culture. The detailed procedure is described by Cristofalo and Charpentier (4).

**Indirect Immunofluorescence Microscopy:** For staining with polyclonal antibodies, I followed the protocol established in our laboratory, which introduces a minimum of structural distortion for IF structures. The detailed procedure was described previously (30). For staining with rabbit polyclonal antibody, fluorescein-conjugated goat anti-rabbit immunoglobulin (IgG) was used as the secondary antiserum (Cappel Laboratory, Cochranville, PA). For the removal of nonspecific background staining activity, this secondary anti-serum was absorbed with formaldehyde-fixed and acetone-extracted cell specimens before use. The concentration of IgG used for each staining reaction was standardized.

For staining with mouse monoclonal antibody, cell specimens were prepared in a similar fashion to the procedure described for polyclonal antibodies, except that the initial incubation period of primary antibody was 12–16 h at 37°C to ensure the binding reaction between IgG molecules and the cytoskeletal proteins. The second antiserum used for staining monoclonal antibodies, fluorescein-conjugated goat anti-mouse immunoglobulin (IgM) was used as the secondary antiserum (Cappel Laboratory, Cochranville, PA). For the removal of nonspecific background staining activity, this secondary anti-serum was absorbed with formaldehyde-fixed and acetone-extracted cell specimens before use. The concentration of IgG used for each staining reaction was standardized.

Prepared samples were examined with a Zeiss photomicroscope III equipped with epifluorescence illumination (Carl Zeiss, Inc., Thornwood, NY). A Zeiss mercury HBO 50 arc lamp with a proper combination of filters was used as the light source. Photomicrographic recording was performed with Kodak Tri-X film at ASA 800, and processed with Kodak D safelight. For staining with mouse monoclonal antibody, cell specimens were prepared in a similar fashion to the procedure described for polyclonal antibodies, except that the initial incubation period of primary antibody was 12–16 h at 37°C to ensure the binding reaction between IgG molecules and the cytoskeletal proteins. The second antiserum used for staining monoclonal antibodies was fluorescent goat anti-mouse IgG, directed toward both the IgG and IgM molecules of the immunoglobulin family. This secondary antiserum was also preabsorbed with fixed and extracted cells to remove nonspecific binding activity. The concentration of IgG used was ~0.04 mg/ml.

| Repository | Donor age | Donor sex | CPDL | Mean | Range |
|------------|-----------|-----------|------|------|-------|
| GM0011     | 8 fetal wk| M         | 65   | 57–72|       |
| GM2936B    | 20 d      | M         | 57   | 54–60|       |
| GM0038A    | 9 f       | F         | 56   | 52–61|       |
| GM2912A    | 26 yr     | M         | 31   | 27–38|       |
| GM3529     | 66 yr     | M         | 28   | 24–32|       |

Since subpopulations of different phenotypes were present in senescent cultures, it was essential to identify cells according to phenotype. The following protocol was designed for sectioning specifically those cells of interest. After being embedded in Epon, the cells that showed features of aging such as enlargement in cell size and increase in cytoplasmic filaments, which can be observed at the light microscopic level, were selected with an inverted microscope and marked with a fine needle attached to a Leitz micromanipulator. These regions were cut out and mounted on the ends of plastic blocks for sectioning. The sections were mounted on paraffin- and carbon-coated grids, stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

**Indirect Immunoelectron Microscopy:** Colloidal gold particles, ~5 nm in diameter, were prepared according to the procedure described by Faulk and Taylor (6). These particles were then conjugated with protein A (Sigma Chemical Co., St. Louis, MO) or goat anti-rabbit or anti-mouse IgG following the procedure for gold–protein conjugation described by Horisberger et al. (10).

Localization of the position of antigen-antibody interaction with the gold–protein A complex was performed by a procedure similar to that for immunofluorescence microscopy. Initially, cells were grown on Thermopax plastic coverslips (Lab-Tek Co., Naperville, IL) for 48–72 h. The cells were then processed for fixation with formaldehyde and acetic extraction, as described previously (32). After they were rinsed with phosphate-buffered saline (PBS) that contained bovine serum albumin (BSA, 5 mg/ml), the samples were incubated with primary antibody at 37°C for 2 h, then rinsed with PBS that contained BSA. The samples were then incubated with 5 nm colloidal gold–conjugated protein A, or colloidal gold–conjugated goat anti-rabbit or anti-mouse IgG for another 2 h before the final rinsing with PBS that contained BSA. The samples were then processed for electron microscopy as described above.

**Immunoprecipitation:** Cultures of 0011 and 3529 cell strains at both low and high CPDLs were washed in leucine-free medium and incubated at 37°C for 15 min before the addition of tritiated leucine (200 μCi/mol, New England Nuclear, Boston, MA). Approximately 5 ml of medium per 100-mm Petri dish was used during labeling for 16 h. At the end of the labeling period, the cultures were washed again with warm PBS, pH 7.2.

Labeled cytoskeletal fractions were immunoprecipitated by modification of the protein A method of Kessler (12). Briefly, 2 μl undiluted rabbit polyclonal antibody to vimentin was mixed with 10 μl of the labeled extract which had been adjusted to 0.1% SDS and 0.5% Nonidet-P40. The mixture was then incubated at 37°C for 5 h. Antigen–antibody complexes were precipitated with 40 μl of a 1:10 (vol/vol) suspension of protein A Sepharose beads. To minimize protease activity, I included phenylmethylsulfonyl fluoride at 0.5 mg/ml in all steps. The immunoprecipitates were washed and boiled for 5 min in 0.0625 M Tris-HCl, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and analyzed by electrophoresis and autoradiography (11).

**Time-lapse Cinematographic Studies of Cell Locomotion:** Fibroblast cultures at early and late CPDLs of two cell strains (0011 and 3529) were planted in 25-cm² Falcon flasks (Falcon Labware, Oxnard, CA). The flasks were equilibrated with 5% CO₂ in air. Cultures were then photographed at 2-min intervals under phase-contrast optics with a 10x objective. The developed films were projected, and the movements of individual cells over a period of 72 h were traced onto graph paper. The rate of cell translocation across the plastic substratum was then analyzed as illustrated in Table II.

**RESULTS**

Cell cultures of the five different strains under study were trypsinized and seeded at a density of 3,550 cells/cm² growth

**Table II**

| Cell strains | CPDL | Frequency of locomotion | Rate of locomotion (μm/min ± SD) |
|--------------|------|------------------------|---------------------------------|
| 0011         | 8    | 88% (45/51)            | 0.1359 ± 0.0078                 |
| 0011         | 60   | 43% (48/111)           | 0.0349 ± 0.0014                 |
| 3529         | 8    | 73% (67/92)            | 0.1041 ± 0.0109                 |
| 3529         | 30   | 32% (34/106)           | 0.0215 ± 0.0091                 |

* The proportion of individual cells engaged in movement in 72 h was analyzed directly on the developed film with a stop motion projector. The paths were traced with a distance tracking device, and the measurements were divided by the total magnification.

* Mean value of determinations derived from four separate experiments.

* The number of cells engaged in movements over the total cell number examined.
area. As the cultures grew into confluence, the cells were harvested, and cell number was determined by direct cell count. Population increase in terms of doublings per passage was calculated as follows: \( PDs = \log(N_h) - \log(N_i)/\log2 \), where \( N_h \) is the cell number at harvest, and \( N_i \) is the initial cell number. The increase in population doublings was then added to the previous population doubling level to give the CPDL (4).

Subcultivation of cell cultures for the five cell strains was performed on a weekly schedule, provided that the culture reached confluence within 1 wk. If, after re-feeding and culturing for 3 wk, the cells were not confluent, the culture was considered to have phased out if \( > 14 \) d were required for the population to double. The CPDL at phase out was considered to represent the replicative life span of a cell strain. This standard of evaluation was described in detail by Cristofalo and Charpentier (4).

We have performed four separate successful longitudinal studies according to the above procedure. The CPDL for each of the five cell strains is listed in Table I. The pattern of their in vitro life history is demonstrated in Fig. 1. A “window” period of sharp increase in days required for population to double was observed in all five strains close to the end of their in vitro life span.

As commonly reported in the literature, human fibroblasts in culture commonly display considerable heterogeneity. In the early part of their in vitro life, the cell culture of the five strains of fibroblasts contained predominantly small and spindle-shaped cells. As the culture became senescent, the proportion of this subpopulation became diminished and the subpopulation of cells that displayed an enlarged and flattened morphology became predominant. In cultures derived from older donors, the subpopulation that contained the large cells was already found in a significant fraction (5–10%) in the initial period of their in vitro life span, whereas large cells constituted a very small proportion (0.1–0.5%) of the cell population for the 0011 cell strains (derived from a donor of 8 fetal wk).

Immunofluorescence microscopy, using rabbit polyclonal antibody to vimentin, demonstrates that in young, rapidly dividing fibroblasts, IFs are organized as a fibrous network composed of individual filaments distributed from the nuclear region to the cell periphery (Fig. 2a). This network of separate filaments is observed in all five fibroblastic cell lines at the early stages of their in vitro life span, when the rate of proliferation is high. In contrast, vimentin in those large cells at later stages of in vitro life span is observed as bundles compacted into the tightly aligned filaments (Fig. 2b). The bizarre IF formation into bundles, sometimes observed as aggregates of hundreds of filaments coursing through the cytoplasm, is observed in all five cell strains.

At the ultrastructural level, the aging-related conformational alteration in the IF organization was even more dramatic. In young fibroblasts of 0011 cell strain (CPDL = 8), microtubules and IFs are seen as interdispersed arrays with various organelles distributed in different locations (Fig. 3A). In similar cytoplasmic areas, senescent fibroblasts with CPDL > 60 contain IFs as compacted bundles of fibers which exclude most organelles except mitochondria and vesicles (Fig. 3B). At higher magnification, the contrast between the microtubule–filament complexes in young cells (Fig. 4A) and the filament bundles in old cells is more obvious, and cross-bridges along the individual filaments are distinct (Fig. 4B). These cross-bridges are observed mostly as side-arm structures which extend perpendicularly to the filament core.

A similar contrast between young and senescent fibroblasts in their distribution of IFs can be observed with immunofluorescence staining, using mouse monoclonal antibody to an

![Figure 1](image1.png)  
**Figure 1** The replicative life span of human diploid skin fibroblasts derived from donors of varying ages: 0011 (8 fetal wk), 2936B (20 d), 0038A (9 yr), 2912A (26 yr), and 3529 (66 yr). The maximal CPDL is calculated as described in the text. Briefly, the population doubling levels (PDL) were estimated by comparison of the cell numbers at seeding with cell numbers at harvest. The addition of the PDs to the number of the previous population doubling level gives rise to the CPDL. The number of days required per doubling is estimated by the time spent during each PDL.

![Figure 2](image2.png)  
**Figure 2** Fluorescence microscopy of the distribution of IFs in young and senescent fibroblasts by staining with rabbit polyclonal antibody to vimentin. (a) Vimentin staining pattern in young replicative fibroblasts of the 0011 cell strain at CPDL = 9. A similar staining pattern of vimentin has been observed in young cells of the other four cell strains. (b) 0011 at CPDL = 67. The vimentin-containing filament profile shown in this micrograph is not restricted to the 0011 cell strains. In fact, this pattern of filament distribution can be found in different senescent fibroblasts within the same strain of fibroblast culture. × 720.
IF-associated protein, p50 (30). As shown by Fig. 5a, antibody to p50 stains a typical IF distribution pattern in young cells of all five fibroblast strains. The bundle formation of IFs, as seen by staining with antibody to vimentin, is also observed with the cytoplasmic distribution of p50 (Fig. 5b).

Immunogold localization with polyclonal rabbit antibody to vimentin also demonstrates the bundling configuration of IFs in the enlarged senescent fibroblasts (Fig. 6, a and b).
FIGURE 4 A higher magnification of structural profiles similar to those shown in Fig. 3. (A) Young (CPDL = 9) and (B) senescent (CPDL = 67) fibroblasts. The side-arm structures (arrows) of IFs in B are most evident. X 50,000.

Arrays of linearly aligned gold particles, which represent the location of vimentin, disperse throughout the cytoplasm. Since the antibody reacts with the protein monomers that form the filament core, the dendritic branching out of individual filaments from the bundles is clearly illustrated by many gold particle-decorated fibers. In contrast, the labeling of an IF-associated protein recognized by a mouse monoclonal antibody shows that the protein, p50, is observed between the filaments, possibly reflecting interfilament links (Fig. 6c). This type of orderly arranged bonds via gold-decorated IF-associated proteins among the filaments is rarely observed in young cells, where most IFs are seen to be farther apart from
DISCUSSION

I have demonstrated the existence of large, densely packed bundles of IFs in nonreplicating senescent fibroblasts. The permanent configuration of IFs as large bundles may be indirectly related to the absence of mitosis-associated total cytoplasmic rearrangement. However, I hypothesize that the formation of these enormous bundles is not due to the accumulation of more vimentin subunits in the large senescent fibroblasts, but rather to the increased cross-linking interaction among the filaments. I present here evidence that an IF-associated protein, p50, may function as one of these cross-linking structures that establishes and maintains the IFs in this large bundled form. Furthermore, the presence of IFs as large bundles may indicate a reduction in the ability of rapid disassembly and assembly of subunits, which impairs the flexibility of network reorganization for these filaments. The phenotypic expression of such impairment may therefore be manifested as the reduction in cell locomotion presented in Table II.

Recently, the cessation of replication at the end of the fibroblasts’ in vitro life has been hypothesized to be regulated by the expression of a dominant set of genes that stops the traverse of cell cycle at the Go/G1 phase (1, 15, 16, 25). The organization of IFs into unusually large bundles in nonreplicative cells is a phenotypic manifestation after the termination of cell proliferation and the absence of drastic cytoplasmic rearrangement associated with cell division.

A permanent organization of IFs has been observed in three other types of IFs in various tissue systems. For example, a permanent scaffold of tonofilaments, composed of a unique set of keratin proteins, has been commonly observed in cells of epithelial origin at the terminal stage of their differentiation (27). The formation of glial fibrillary acidic protein in long bundles traversing long cellular processes is the necessary feature for the stellate morphology of astrocytes (21). Finally, the well-organized bundles of neurofilaments in axonal processes may represent the most specialized organization of IFs (17, 22–24).

The organization of IFs into large bundles can be simply due to the accumulation of more cellular materials in the large nondividing cells. However, I have compared the organization of IFs in senescent cells with that in other nonproliferative cells produced under conditions such as serum starvation or treatment with interferon (18, 19). In both cases, fibroblasts fail to divide and, to a certain extent, cell gross morphology becomes enlarged, flattened, and spread over the substratum. However, other than an increase in the number of filaments per cell, the organization of IFs may be viewed as simply the larger image of those seen in dividing cells. Therefore, I hypothesize that an additional regulatory mechanism is at work to link the accumulated individual filaments into large bundles in the nonproliferating senescent fibroblasts.

Cross-bridging structures have been the popular hypothesis for interfilament links suggested as the regulatory mechanism needed for defining either the spatial distance, topographic arrangement, or mechanical strength within the large IF bundles. Recently, a number of IF-associated proteins have been identified and postulated to serve such functions. Perhaps the most well-defined IF-associated protein is the 200,000-D protein of the neurofilament triad. Evidence from both biochemical and immunocytochemical results suggests the carboxyl
FIGURE 6 Immunogold labeling pattern of vimentin and associated protein p50 in nonreplicating senescent fibroblasts of 0011 cell strain at CPDL = 70. The detailed procedure is described in Materials and Methods. (a and b) Staining with polyclonal antibody to vimentin clearly demonstrates the dendritic pattern of filaments within each bundle. Fig. 6a shows the higher magnification of the rectangle area marked in 6b and illustrates the gold particles positioned directly on the filaments. (c) Staining with mouse monoclonal antibody to p50 illustrates the close physical affiliation of filaments within a given bundle, by the neatly arranged clusters (arrow) of gold particles dispersed among the filaments. (a) x 54,000; (b) x 32,350; (c) x 53,000.

terminal of p200 as the possible cross-bridging structure that connects neurofilaments laterally in nerve axons (7, 9, 14). A similar candidate for such function is filaggrin, which functions as the bundling protein for keratin polypeptides in terminally differentiated keratinocytes (5). Here, we suggest a protein p50, otherwise named epinemin, which may serve similarly to connect the filaments into large bundles in non-dividing senescent fibroblasts (13, 30).

Many laboratories are engaged in intense study of how these proteins function to link the filaments physically. Evidence has begun to emerge that phosphorylation may play a key role in pivoting the protein to interact with the core protein of the filaments (35). Much biochemical study will be needed to elucidate the detailed action of phosphorylation in the actual mechanism of cross-bridging.

In addition to the obvious function for interfilament linkages, cross-bridges have also been related to various motile activities. The most convincing evidence is that the increase in the p200-related cross-bridging structures is thought to be involved in the decline in the axonal transport during development or aging (reference 32; Lasek, R. J., and I. G. McQuarrie, personal communication). Here, I suggest that although the large IF bundles may be the architecture needed to maintain the permanent enlarged morphology of senescent fibroblasts, the acquisition of this permanence then reduces the plasticity required for movement across the substratum. It is not surprising then that the motile activity of aged fibroblasts is significantly decreased. The regulatory mechanism needed for the formation of large IF bundles may at the same time restrict the possibility of an interplay between
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dismantling and construction of the IF network required for changes of cell shape during locomotion. Whether we observe here in the in vitro environment can be used to explain the slower rate of wound healing in older individuals requires further in vivo comparative studies of IF organization and cell locomotion.

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