Ultrastructural immunolocalization of the senescence-related protein terminin in human fibroblasts

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Summary

Terminin is a cytoplasmic protein originally found in human fibroblasts, where it can be present in three forms of different molecular weights: the 90 kDa form is synthesized in young fibroblasts, and is cleaved into a 60 kDa form in irreversibly growth-arrested senescent cells; a 30 kDa form of terminin is found in apoptotic cells. All the forms can be immunodetected by monoclonal antibody 1.2 in Western blots, whereas in immunohistochemistry the same antibody only recognizes the insoluble 60 and 30 kDa forms. Terminin immunopositivity may, therefore, be considered as a marker for cell senescence/ageing, terminal differentiation, and commitment to apoptosis, although the role(s) of this protein and its cleavage products in cell metabolism are still unknown. In the present investigation we aimed to elucidate the intracellular distribution of terminin in senescent human fibroblasts, using a combined immunohistochemical approach of light and transmission electron microscopy. For light microscopy, we used indirect immunodetection methods, utilizing either fluorochrome-labeled secondary antibodies or horseradish peroxidase (HRP)-conjugated antibodies finally revealed by incubation with diaminobenzidine (DAB); for transmission electron microscopy, we used either pre-embedding immunodetection with HRP-DAB or post-embedding gold-immunolabeling. All methods consistently identified cytoplasmic vacuoles as the main site of accumulation of immunodetectable terminin; based on their ultrastructural morphology, these membrane-bounded organelles are likely to be phagolysosomes or residual bodies.

Key words: Terminin, immunolabeling, light microscopy, electron microscopy, human fibroblasts.

Introduction

Terminin is a protein expressed in irreversibly growth-arrested senescent human fibroblasts, and recognized by a unique monoclonal antibody (mAb 1.2) (Wang and Tomaszewski, 1991). By immunoblotting, this antibody identifies terminin in the detergent-soluble fractions of young growing and non-growing quiescent fibroblasts as a 90kDa band, whereas in cell extracts from senescent fibroblasts the protein appears as a 60kDa band, in the detergent-insoluble fractions; a further 30kDa band is detected in the detergent-insoluble fractions of apoptotic cells. It is likely that terminin is constitutively synthesized as the 90kDa form, and cleaved to the lower molecular weight forms depending on the physiological conditions, i.e., to the 60kDa form in senescent cells, or to the 30kDa form in apoptosis-committed cells (Wang and Liu, 1996). By immunohistochemistry, this antibody recognizes only the insoluble 60kDa and 30kDa protein forms, resulting in a granular labeling exclusively located in the cytoplasm, whereas no diffuse signal has ever been observed in the cytoplasm or in the nucleus (Wang and Tomaszewski, 1991).

Using mAb 1.2, terminin labeling has been detected not only in senescent cultured fibroblasts, but also in terminally differentiated cells of rodent skin and duodenal epithelia (Wang and Tomaszewski, 1991), in differentiated neurons of rat brain and cornea (Yang and Wang, 1993), and in aged neurons and glia of humans and rats (Yang and Wang, 1994;

Schipper *et al.*, 1994). Moreover, terminin has been found in the rat prostate after castration (Mitmaker *et al.*, 1993), in the mouse regressing uterus (Miller *et al.*, 1995), in cultured fibroblasts experimentally induced to undergo apoptosis (Hébert *et al.*, 1994), and in human myoblasts aged in vitro (unpublished personal observation).

Terminin immunopositivity may, therefore, be considered not only as a marker for cell senescence, but also for cell terminal differentiation, ageing and commitment to apoptosis (Wang et al, 1994), with obviously wide potential applications in the biomedical and pathophysiological fields, although the roles of this protein and its cleavage products in cell metabolism are still unknown.

In the present investigation we aimed to elucidate the intracellular distribution of terminin, as a necessary step toward understanding its function; to do this, we analyzed senescent human fibroblasts using a combined immunohistochemical approach of light and transmission electron microscopy (TEM).

## Materials and Methods

Cell culture

Asynchronously growing primary cultures of normal human dermal fibroblasts at the fifteenth passage were grown on glass coverslips in multi-well plates in DMEM (Dulbecco Modified Eagles Medium), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 units/mL of streptomycin and penicillin.

In order to provide *in situ* evidence for the presence of senescent fibroblasts, beta-galactosidase was detected according to Dimri *et al.* (1995); in the cell cultures used, about 87% of the fibroblasts proved to express beta-galactosidase (Figure 1).

Immunolabeling

Immunofluorescence

Cells on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at 4°C, then post-fixed for 30 min with 70% ethanol at -20°C (for membrane permeabilization), rehydrated in PBS, incubated overnight with mAb 1.2 at 4°C, and finally revealed with an Alexa488-conjugated anti-mouse IgG antibody (Molecular Probes, Invitrogen, Milan, Italy) for 60 min at room tem-
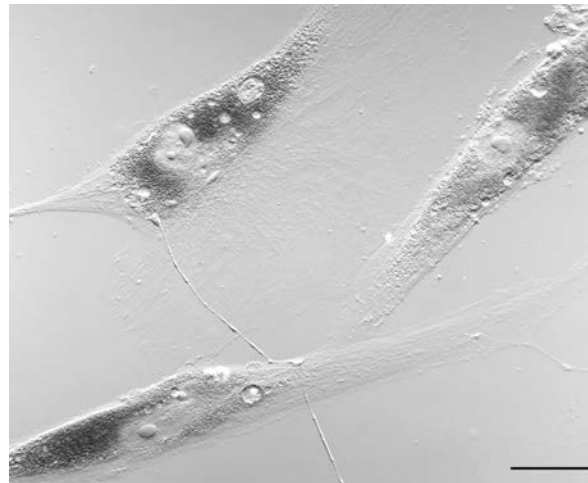
Figure 1. Detection of beta-galactosidase (dark precipitates) in the cytoplasm of fibroblasts. To make the cell boundary and nucleus visible, the micrograph was taken by DIC microscopy. Bar = 30 μm.

perature. Some immunolabeled preparations were also counterstained for DNA with Hoechst 33258 (Sigma, Buchs, Switzerland; 1 μg/mL for 10 min), and were mounted in a drop of Mowiol (Calbiochem, Milan, Italy).

An Olympus BX51 microscope equipped with a 100W mercury lamp was used under the following conditions: 450-480 nm excitation (excf) filter, 500 nm dichroic mirror (dm), and 515-550 nm band-pass filter for Alexa 488; 330-385 nm excf filter, 400 nm dm, and 420 nm long-pass filter, for Hoechst 33258. Images were recorded with a Camedia 5050 digital camera system, and stored on a PC by Olympus software, for processing and printing.

Horseradish peroxidase immunolabeling for light and electron microscopy

Cells on coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at 4°C, pre-incubated with 0.03% H_2O_2 for 10 min (to block endogenous peroxidases) and incubated with mAb 1.2, as above; as a secondary antibody, a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody was finally revealed by incubation with 3,3' diaminobenzidine (DAB) (25 mg/100 mL in PBS), in the presence of 0.003% hydrogen peroxide, for 30 min at room temperature. Coverslips were then incubated with 1% OsO_4 for 60 min at room temperature. As a control, some cell preparations were treated as described above, omitting the incubation with the primary anti-terminin antibody.

For light microscopy, stained cell preparations were mounted upside down on glass slides in a drop of Eukitt (Bioptica, Milan, Italy). Slides were observed and photographed with the same microscope and digital camera utilized for fluorescence microscopy, using differential interference contrast (DIC) to allow simultaneous visualization of cell boundaries and nuclei.

For TEM, HRP-DAB-stained cell monolayers were dehydrated with acetone, and embedded in Epon resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections (80-90 nm in thickness) were placed on copper grids coated with a formvar layer (Electron Microscopy Sciences, Hatfield, PA, USA), conventionally stained with uranyl acetate and lead citrate, and observed in a Philips Morgagni TEM operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

Immunogold labeling for electron microscopy

Cell monolayers were fixed with a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M Sörensen phosphate buffer at 4°C for 2 h, washed, treated with NH_4Cl 0.5 M in PBS for 45 minutes, dehydrated with ethanol and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections (80-90 nm in thickness) were placed on nickel grids coated with a formvar-carbon layer, and processed for immunocytochemistry with the same anti-ter-

minin antibody used for fluorescence microscopy. Sections were floated on normal goat serum (NGS) diluted 1:100 in PBS, and incubated overnight at 4°C with the primary antibody diluted 1:100 in a solution containing 0.1% BSA and 0.05% Tween 20 in PBS. After rinsing, sections were floated in NGS and then reacted for 30 min with the specific 12 nm gold-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:10 in PBS. Finally, the sections were rinsed and air dried. As a control, some grids were treated with the incubation mixture without the primary antibody, and then processed as described above.

The sections were weakly contrasted with uranyl acetate, and then observed in the Philips Morgagni TEM, as above.

Results

Immunofluorescence

In the senescent fibroblast cultures used for this study, more than 90% of the cells proved to be positive for terminin: the immunopositivity appeared in the form of bright granules dispersed in the cytoplasm, whereas the nucleus was devoid of labeling (Figure 2, left panel); a ring-shaped fluorescence pattern around a dark core was often observed (inset in the left panel of Figure 2).
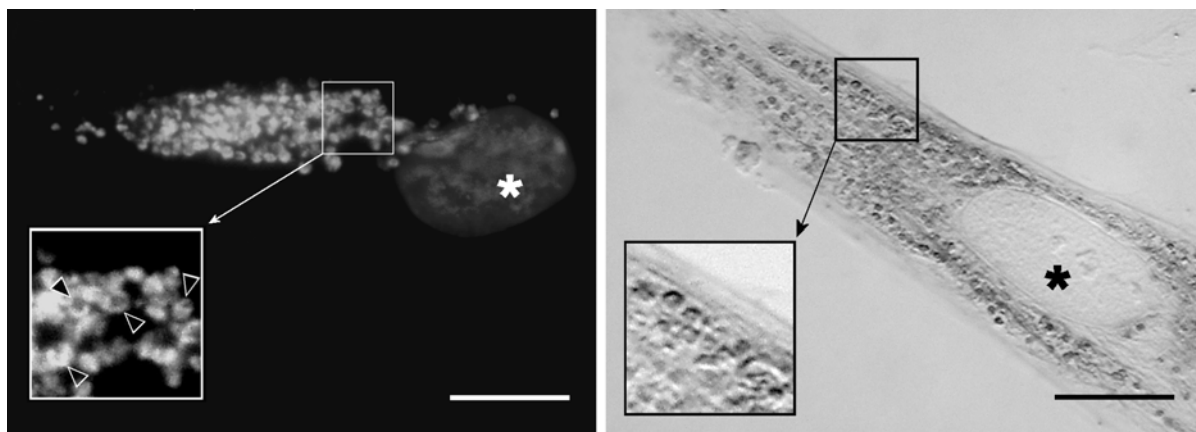

Figure 2. Left panel: Immunofluorescence labeling of terminin in a senescent human fibroblast. Terminin is exclusively located in the cytoplasm, and often appears in ring-shaped, brightly fluorescing spots (arrowheads in the inset). The nucleus (asterisk) was weakly counterstained with Hoechst 33258. Bar = 20 μm. Right panel: Terminin immunopositivity in a senescent human fibroblast after the HRP-DAB procedure followed by OsO_4 treatment. The localization and morphology of terminin granules resembles that seen after immunofluorescence labeling. DIC microscopy; the nucleus is marked by an asterisk. Bar = 20 μm.

Horseradish peroxidase immunolabeling for light and electron microscopy

Under light microscopy, terminin immunoreactivity was identified by a granular brown reaction product in the cytoplasm of senescent fibroblasts (Figure 2, right panel). Under TEM, an electron-dense reaction product was found in numerous cytoplasmic vacuoles, which appeared heterogeneous in both size and content: most of them showed electron-lucent areas surrounded by a peripheral layer of electron-dense granular material (Figure 3a-c), while others contained cellular
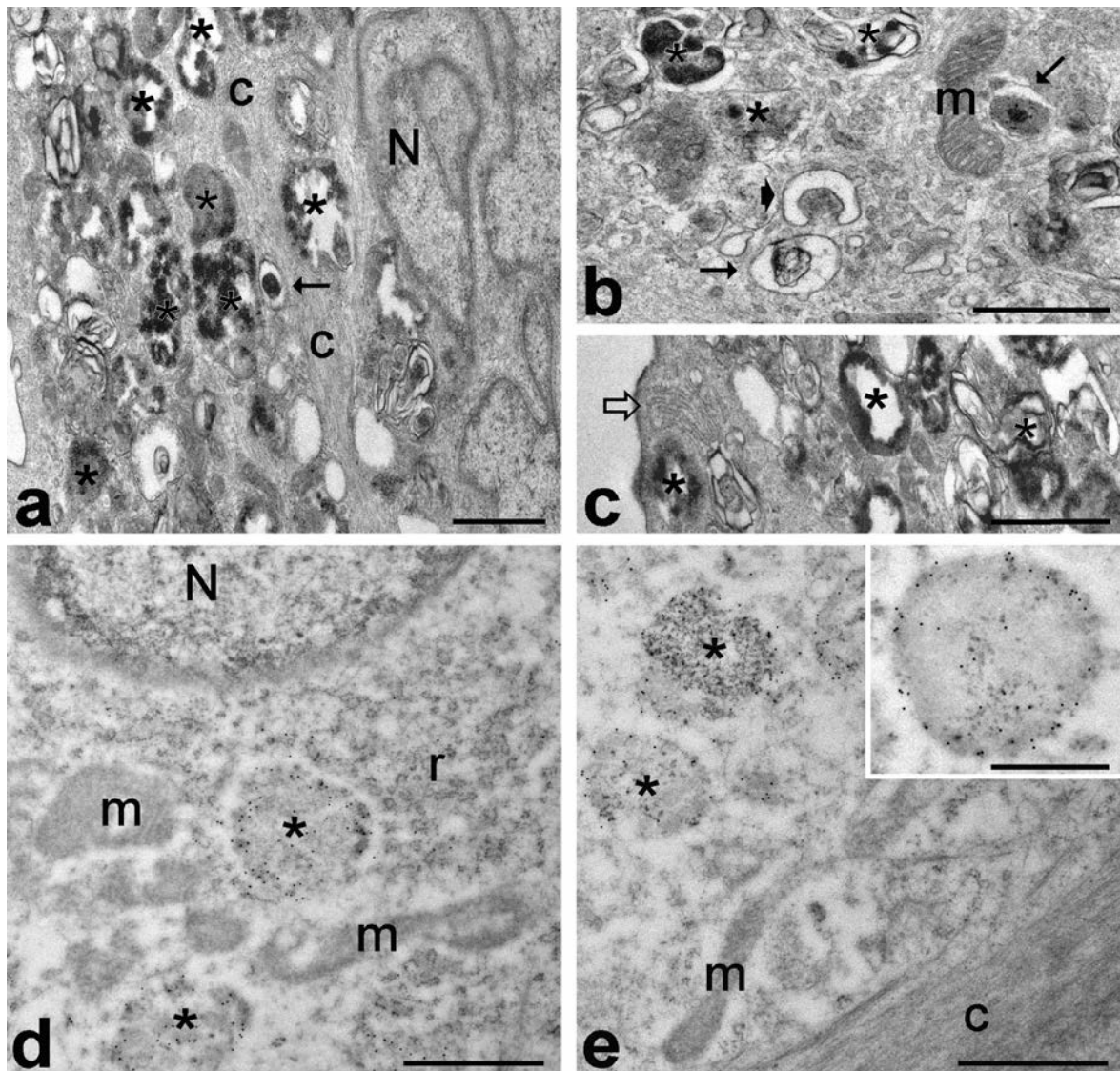
Figure 3. Electron micrographs of senescent fibroblasts. a, b, c: Pre-embedding immunolabeling of terminin, DAB reaction. The electron-dense reaction product is present inside heterogeneous vacuoles (asterisks), as well as in cellular debris enclosed by membranes (arrows), and often accumulates at the periphery of vacuoles. No labeling is present over the cell nucleus (N), cytoskeletal bundles (c), mitochondria (m) or the Golgi apparatus (open arrow). Note the pincer-shaped cisterna partially enclosing a DAB-positive cytoplasmic region (large arrow in b). d, e: Post-embedding immunolabeling of terminin, 12 nm colloidal gold grains. The labeling is mostly located in vacuoles characterized by heterogeneous content (asterisks); here too the gold grains are especially numerous at the vacuole periphery (inset in e). Weak labeling for terminin also appears in a diffuse form in the cytoplasm, in close proximity to ribosome clusters (r). The cell nucleus (N), mitochondria (m) and cytoskeletal bundles (c) are devoid of gold grains. Bars = 1 µm; Bar in the inset = 0.5 µm.

debris or membrane fragments (arrows in Figure 3a, b). No other cytoplasmic organelles were HRP-DAB-positive, whereas a finely dispersed reaction product was observed in limited areas of the cytosol: here, short ring-shaped cisternae, reminiscent of initial autophagic vacuoles, sometimes surrounded the cytosolic DAB clumps (large arrow in Figure 3b). No labeling was ever found in the nucleus.

In control samples, a negligible signal was always present (*not shown*).

Immunogold labeling for electron microscopy

Due to the fixation/embedding procedure (necessary for optimizing antigen preservation), the ultrastructural morphology of fibroblasts was relatively poor in LR White-embedded samples; however, despite the lack of well defined cellular membranes, all the cellular organelles were clearly recognizable. Consistent with the observation in HRP-DAB-labeled samples, the gold grains revealing terminin were mostly located in vacuoles containing heterogeneous material (Figure 3c, d); in addition, a dispersed weak cytosolic positivity was also found, especially in ribosome-rich areas.

Conclusions

This study describes, for the first time under electron microscopy, the subcellular distribution of the senescence-related form of terminin in senescent human fibroblasts.

All the immunocytochemical techniques used consistently identified cytoplasmic vacuoles as the main site of accumulation of the immunodetectable form(s) of terminin.

It is worth stressing that the pre- and post-embedding procedures for terminin labeling provide complementary evidence for the cytoplasmic localization of this protein: in fact, the HRP-DAB technique requires post-fixation with OsO_4, which allows visualization of all the cell membrane systems, thus permitting unequivocal recognition of terminin either inside vacuoles or in the cytosol; on the other hand,

gold immunolabeling is especially sensitive, and makes it possible to detect even very small terminin aggregates, which would not be clearly discernible after the HRP-DAB procedure alone.

The nature of the terminin-containing vacuoles remains to be fully elucidated, but their morphology strongly suggests phagolysosomes and/or residual bodies. It may be hypothesized that, during cell senescence of fibroblasts *in vitro*, the constitutive 90 kDa form of terminin accumulates in lysosomes, and is here cleaved by proteases into the immunodetectable form(s). The fact that weak immunolabeling is also present in the cytosol suggests that the proteolytic cleavage of 90 kDa terminin may be induced by cytosolic proteases as well; the resulting cleaved proteins proved to be also internalized by autophagy in two-membrane bounded vacuoles. The progressive accumulation of immunodetectable terminin during cell senescence would therefore be the consequence of an impairment in cell degradation processes, leading to the accumulation of residual bodies in the cytoplasm of senescent cells.

The ultrastructural immunolabeling techniques we have used in the present study open promising perspectives in the still elusive search for terminin function: they will allow detailed monitoring of the dynamics of appearance, redistribution and accumulation of detectable terminin not only during cell senescence (for which terminin is still an almost unique marker), but also in the commitment to regulated forms of cell death. Detecting terminin in specific subcellular locations after specific death-inducing stimuli would possibly provide clues to elucidate the role of this protein in the various cell death pathways.

Acknowledgements

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