Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1915–1918, April 1983
Cell Biology

Functional changes of intermediate filaments in fibroblastic cells revealed by a monoclonal antibody

(vimentin/immunofluorescence/epitope)

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Contributed by Renato Dulbecco, December 20, 1982

ABSTRACT **We describe reversible changes of intermediate filaments of fibroblastic cells associated with changes in the functional state of the cells. The changes are revealed by comparing the immunofluorescence patterns given by a monoclonal antibody and a polyclonal serum, both recognizing vimentin. The state of the filaments depends on culture density; this effect cannot be attributed to the nutritional state of the cells, their growth rate, or substances released into the medium. It seems to depend mainly on the aggregation of filaments during strong cell movements. The possible significance of these findings for the functional role of intermediate filaments is discussed.**

Intermediate filaments, 8–10 mm in diameter, are present in most cells. They are made up of different proteins that share some common epitope (1). In fibroblastic cells, the main constituent of the filaments is vimentin (2, 3), with a molecular weight of 58,000; smaller related peptides (molecular weight, >40,000) are also observed (4, 5) and are thought to be cleavage products of vimentin by a specific protease (6). Vimentin molecules associate to form protofilaments: various models have been proposed for this association (7, 8). Protofilaments are associated in bundles; in muscle cells and nucleated erythrocytes, they are crosslinked by another protein, synemin (9, 10); protofilaments are aggregated by cationic proteins (11). Vimentin undergoes phosphorylation, which may have a regulatory significance (12, 13). Under the action of colchicine, intermediate filaments collapse into a coiled mass near the nucleus (14); collapse is also caused by intracellular injection of a monoclonal antibody to a common antigen of the intermediate filaments (15) and, in lymphocytes, by capping of surface molecules (16). This and other evidence shows that intermediate filaments are connected to the plasma membrane (10, 17). They also form a cage around the nucleus (18) and appear to be connected to the nuclear membrane (19).

The role of intermediate filaments is not clear. They are not strictly required for cellular movements (15). A generally accepted idea is that they locate the nucleus in the cytoplasm (20). It is not easy to see, however, how they perform such a function in cultured cells during their sharp changes in shape. If they perform this function, they must be very plastic.

In this article, we report that intermediate filaments are plastic and that plasticity is related to cell movement. These results were obtained by taking advantage of a monoclonal antibody that was raised against Thy-1 and has Thy-1.1 specificity (21). This antibody crossreacts with the intermediate filaments of fibroblastic cells (22). We have found that this crossreactivity varies greatly in the same cell type, depending on the state of the cultures. The variations appear to be caused by a rearrangement of vimentin and to be related to cell movement.

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MATERIALS AND METHODS

NIH 3T3 cells were obtained from J. Cooper and F2408 cells were obtained from W. Eckhart. They were maintained in Dulbecco-modified Eagle medium/10% calf serum and were transferred twice a week. The T11A9e hybridoma (IgM) was obtained from P. Lake and E. Clark; the antibody was used as serum of mice inoculated intraperitoneally with hybridoma cells. The vimentin rabbit antiserum was a gift of J. Singer. For immunofluorescence, cells were fixed for 1 min in cold (−20°C) methanol/acetone (1:1) and then rehydrated in phosphate-buffered saline. T11A9e mouse and rabbit antivimentin sera were used as 1:30 and 1:200 dilutions, respectively. Immunofluorescence was carried out by the sandwich technique using a fluorescein-conjugated goat serum for the second layer. With T11A9e, a third layer of fluorescein-conjugated rabbit anti-goat serum was used to enhance fluorescence. The preparations were examined under epi-illumination. Some cultures were labeled with [^{3}H]thymidine at 2 μCi/ml (1 Ci = 37 GBq) for 2 hr; they were then fixed in neutral formalin for 20 min, washed in phosphate-buffered saline, and dried. They were overlaid with photographic emulsion, developed after 1 wk, and counterstained with hematoxylin.

Characterization of Material Stained by Hybridoma T11A9e. The material stained by T11A9e was characterized by the immunological blotting technique. Cytoplasmic extracts of sparse or confluent NIH 3T3 cultures were prepared (23) by lysing monolayers in 1% (wt/vol) deoxycholate/1% (vol/vol) Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/7 mM NaCl/1 mM $MgCl_2$/100 mM NaF/7 mM Tris·HCl, pH 7.4. The lysates were centrifuged at 4°C for 10 min at 3,000 × *g* to sediment nuclei. The supernatants were run on 10% Na-DodSO_4 gels and blotted to nitrocellulose paper (Schleicher & Schuell; BA85, 0.45 μm) (24). The nitrocellulose paper was incubated in undiluted culture supernatant of T11A9e hybridoma or a 1:30 dilution of T11A9e mouse serum. Bound T11A9e was located by using either ^{125}I-labeled goat anti-mouse Ig or rabbit anti-mouse IgM followed by ^{125}I-labeled protein A. The results were identical. The blots were exposed to XAR-5 x-ray film using intensifying screens.

Time-Lapse Cinematography of Wounds. Confluent monolayers of NIH 3T3 or rat F2408 fibroblasts grown in 3-cm dishes were wounded with a sterile razor blade. Then, the fluid was changed, and the cells migrating from the wounded edge were photographed in an inverted microscope fitted with a 6.3× phase objective using a 16-mm movie camera. Cultures were maintained at 37°C in a CO_2-containing atmosphere and were photographed every 2 min for 24–96 hr. At the end of filming, polaroid photographs of the filmed area were taken before and after fixing the cells in acetone/meth-

anol to monitor for changes in cells during fixing and staining. The wounded area was stained for T11A9e immunofluorescence and photographed. Photographs of the stained cells were matched to the time-lapse film and the movement of individual cells was evaluated by running the film backward.

RESULTS

Preliminary observations showed that the immunofluorescence staining of NIH 3T3 or F2408 cells with T11A9e is brilliant when the cells are sparse but absent when they are crowded. To establish the causes of the difference, we followed the protocol of Fig. 1. Two experiments were carried out with either NIH 3T3 cells or F2408 cells, with very similar results; one set of results with NIH 3T3 cells is given in Table 1. The data show the following: (*i*) In dense cultures (A and D), T11A9e does not stain intermediate filaments whereas it does stain them in all sparse cultures irrespective of their history; (*ii*) the concentration of serum in the culture medium has a minimal effect; and (*iii*) there is no correlation between the thymidine labeling index and the degree of immunofluorescence. In the transfer from dense to sparse cultures (B), cycloheximide (1 μg/ml) was added to the medium of some cultures when the cells were seeded. T11A9e immunofluorescence developed regularly, although cell growth was completely blocked. We did not observe a breakdown of intermediate filaments as described in ref. 25. Some of these variables may have minor effects: fluorescence appears somewhat reduced in cultures transferred from very high density to low density (B, C, E, and F in Fig. 1) or kept for a long

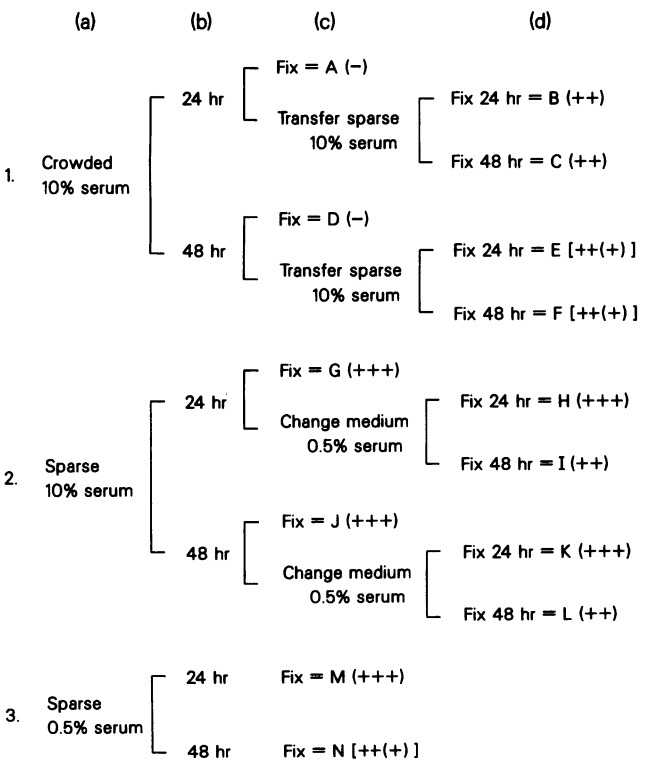

FIG. 1. Protocol of the experiment. Three sets of cultures were prepared as specified in column a (dense = 2.5×10^6 cells per 90-mm dish initially; sparse = 2.5×10^5 cells per 90-mm dish). The cells were incubated as shown in column b and then treated as shown in column c. Cultures that were transferred were further incubated as shown in column d. Each treatment is identified by a letter, A–O. The degree of T11A9e immunofluorescence is shown in parentheses, with the number of + signs being proportional to the brightness. ++(+), Individual cells varied between ++ and +++.

Table 1. Immunofluorescence results

Treatment	Cell density	% serum	T11A9e	LI
A	145	10.0	−	0.30
B	31	10.0	++	0.33
C	67	10.0	++	0.58
D	204	10.0	−	0.42
E	31	10.0	++(+)	0.55
F	51	10.0	++(+)	0.62
G	29	10.0	+++	0.64
H	68	0.5	+++	0.20
I	9	0.5	++	0.21
J	83	10.0	+++	0.53
K	18	0.5	+++	0.22
L	16	0.5	++	0.29
M	40	0.5	+++	0.075
N	8	0.5	++(+)	0.016

NIH 3T3 cultures were subjected to treatments A–N as defined in Fig. 1. Each culture (90-mm dish) contained three coverslips: two were fixed in methanol/acetone and used for immunofluorescence, and one was labeled for 2 hr with [^{3}H]thymidine (2 μCi/ml), then fixed in buffered formalin, overlayed with photographic emulsion, exposed for 1 wk, and developed for radioautography. Cell density is expressed as number of cells per microscopic field with 40× objective counted in the radioautographs. Immunofluorescence is expressed as in Fig. 1. LI, labeling index.

time in 0.5% serum (I, L, and N). The staining with the vimentin antiserum was equal and very bright under all treatments.

These results showed that the staining of intermediate filaments by T11A9e is primarily sensitive to culture density (Fig. 2). The effect of density might be due to cell-produced substances accumulating in the medium. This possibility was ruled out by the results of a wounding experiment—i.e., stripping the cells from a narrow area in a crowded culture. Twenty-four hours after wounding, cells migrating from the wound edge were highly stained by T11A9e, whereas those in the crowded layer adjacent to the wound were stained weakly or not at all (Fig. 3). It was also observed that the staining of cells migrating into the wound was asymmetric, with the forward part

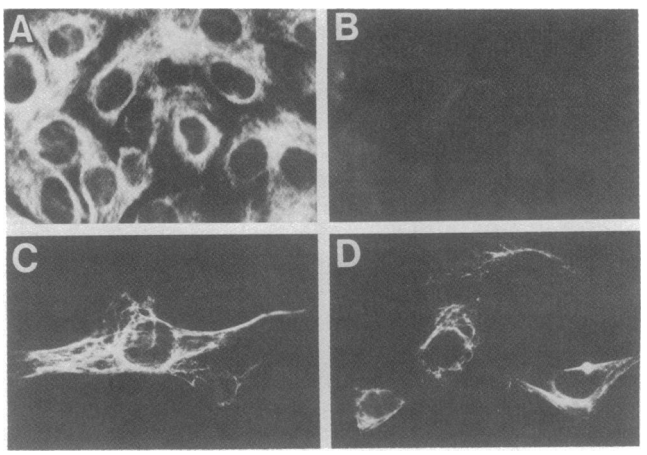

FIG. 2. Some of the immunofluorescence results of the treatments described in Fig. 1. (*A*) Dense cultures (48 hr in 10% serum/medium) were treated with antivimentin serum. (*B*) Cultures as in *A* were treated with T11A9e. Both *A* and *B* correspond to D in Fig. 1. (*C*) Sparse cultures (24 hr in 10% serum) were treated with T11A9e (corresponds to G in Fig. 1). (*D*) Cultures as in *A* were transferred sparse to 10% serum/medium for 24 hr and then treated with T11A9e (corresponds to B in Fig. 1). All cultures gave +++ immunofluorescence with rabbit antivimentin serum.

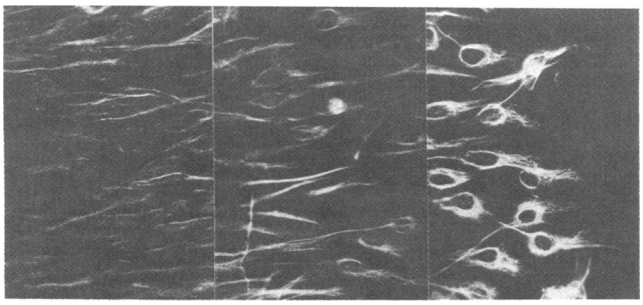

FIG. 3. T11A9e immunofluorescence of three adjacent fields of a culture of NIH 3T3 cells 24 hr after wounding. The left field borders on the dense cell layer, the right field is within the denuded area. The edge of the wound corresponds to the irregular vertical line in the middle field. Note the long bright bridges that begin to appear in the left field and are most pronounced in the central field and the segmentation of intermediate filaments bundles in the left field. The three fields were photographed and printed with equal exposures. (×100.)

of the cell (in the direction of movement) more brightly stained (Fig. 4). This observation suggested that staining is related to the movement of the cells. The same conclusion derives from observing cell colonies: in the dense center, the cells did not stain, whereas they were brilliantly stained at the edges where they are highly motile.

To assess the relationship between immunofluorescence staining and motion, dense cultures were wounded; the cells of a selected area were followed by time-lapse cinematography, and then they were stained for T11A9e immunofluorescence. The results showed a strong correlation between staining and mobility. In crowded cultures, there is little movement and T11A9e immunofluorescence of intermediate filaments is almost nil. In less dense cultures, cells show two patterns of movement: in subconfluent areas, they show a shuffling pattern where the cell edges move slowly and locally; in sparse areas, many cells display a displacement pattern in which the cell body is rapidly shortened and lengthened with extension of long rapidly moving bridges. The shuffling movements are associated with weak T11A9e immunofluorescence of intermediate filaments throughout the cytoplasm; the displacement movements are associated with strong T11A9e immunofluorescence of intermediate filaments around the nucleus and in the moving parts, especially in the extended bridges.

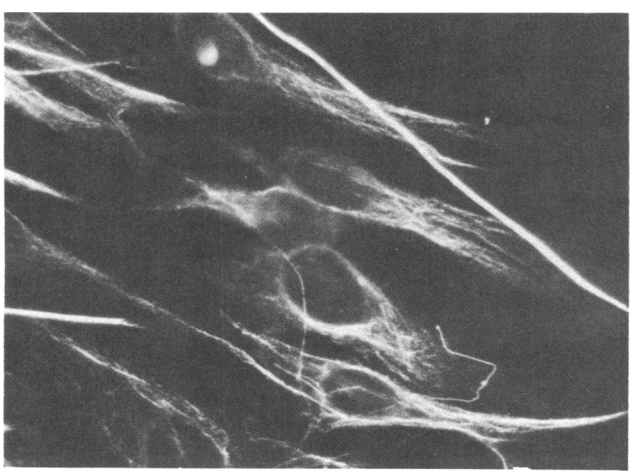

FIG. 4. T11A9e immunofluorescence of NIH 3T3 cells migrating into a wound from left to right. Note the marked polarity of staining, which is higher in the forward part of the cells, and the strongly stained bridges. (×370.)

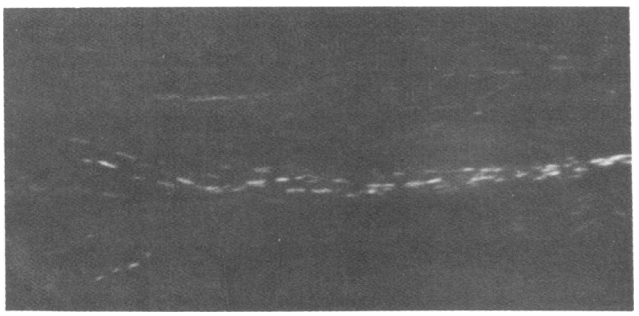

FIG. 5. T11A9e immunofluorescence of a fairly dense culture of F2408 cells. Note the cell bridge containing segmented bundles of intermediate filaments. (×740.)

In areas of intermediate cell density, the immunofluorescence is in short segments, 2 to 3 μm long, or is granular (Fig. 5). Filaments stained by the antivimentin serum were always continuous.

We also observed that, in dense cultures, intermediate filaments collapsed by colchicine (20 μg/ml, 20 hr) were easily stained by T11A9e (data not shown). This result suggests that the crucial factor is the aggregation of filaments to form bundles.

The material stained by T11A9e was characterized by Na-DodSO_4 electrophoresis and binding of the antibody to immunological blots. The antibody identifies a single band of M_r 58,000 in extracts of both dense and sparse cultures, in similar amounts (Fig. 6).

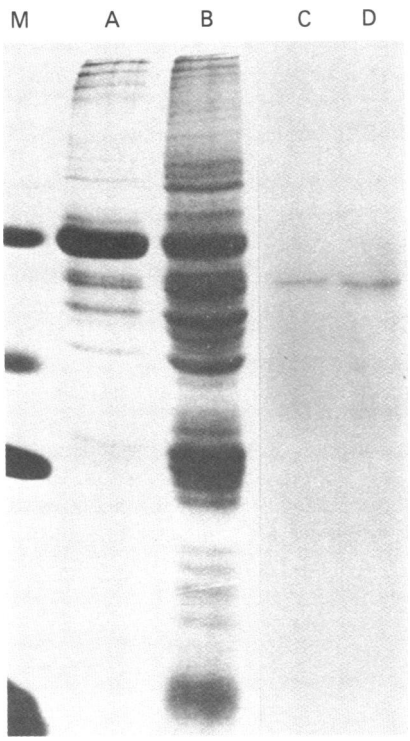

FIG. 6. Cytoplasmic extracts of NIH 3T3 cells were run on 10% Na-DodSO_4 gels, blotted to nitrocellulose paper, and stained with T11A9e. Cytoplasmic proteins from sparse (lane A) and confluent (lane B) cultures were stained with Coomassie brilliant blue. Lane M: M_r markers (bovine serum albumin, 67,000; ovalbumin, 45,000; carbonic anhydrase, 30,000; myoglobin, 18,000). Proteins in sparse and confluent cultures detected by T11A9e and ^{125}I-labeled goat anti-mouse Ig are shown in lanes C and D, respectively. Thy-1 antigen was not detected because NIH 3T3 cells are Thy-1.2.

DISCUSSION

We have observed a striking effect of cell density on the immunofluorescence of intermediate filaments in fibroblastic cells by using the monoclonal anti-Thy-1.1 antibody T11A9e, which crossreacts with a component of the intermediate filaments. Cells of dense cultures are not stained by T11A9e whereas those of sparse cultures stain brilliantly. The effects of cell density are absent when the intermediate filaments are stained with a polyclonal antivimentin serum. We have now shown that in NIH 3T3 cells this antibody binds to a protein that has the molecular weight of vimentin (58,000) and that the amounts are similar in cells from both dense and sparse cultures. These findings suggest that vimentin is equally present in cultures of different densities and that its arrangement within the intermediate filament changes in such a way that the accessibility of the epitope recognized by T11A9e varies. This conclusion is strengthened by the reappearance of T11A9e immunofluorescence when cells are transferred from dense to sparse cultures by trypsinization, even in the presence of cycloheximide, or when the cells migrate from a dense layer into a wound. The vimentin rearrangement is not correlated with the growth state of the cells: T11A9e immunofluorescence does not correlate with the concentration of serum in the medium or with the proportion of nuclei incorporating [^{3}H]thymidine. Time-lapse photographs of cells migrating into a wound show that the intensity of staining with T11A9e correlates with cell movements. The filaments identified by T11A9e fluorescence are sometimes discontinuous, especially in F2408 cells. Cells with discontinuous filaments were present in wounds in an intermediate zone between the dense layer and the rapidly moving cells. This suggests that segmentation or granularity characterize the transition from unstained to stained filaments. The rearrangement of vimentin, therefore, occurs in separate domains along a bundle of intermediate filaments.

It seems that the epitope recognized by T11A9e is not exposed when the intermediate filaments are static but becomes exposed when the filaments elongate and especially when they aggregate in strong bundles. This rearrangement seems to take place only in displacement movements; this is not in disagreement with the results reported in ref. 15; in that study, probably only shuffling movements were observed. Aggregation of filaments by colchicine, in the absence of strong movements, also makes them stainable by T11A9e. It seems likely that rearrangement of vimentin molecules, possibly in conjunction with other proteins, is involved in aggregation under these various conditions.

It has been proposed that the intermediate filaments have the function of locating the nucleus within the cell (20). The present observations suggest that this action is expressed dynamically, through rearrangement of the constituents of the intermediate filament. The functions of these filaments seem to go beyond merely locating the nucleus. Through rearrangement, intermediate filaments are capable of considerable elongation, providing a framework for movements. This framework is not immutable but expands and contracts as the cytoplasm expands. Changes in the intermediate filaments are pronounced during large movements and limited during the formation of "ruffles," which are the basis of the shuffling movement. It is conceivable that the intermediate filaments of neuronal cells undergo similar changes during axonal elongation.

We thank P. Lake and E. Clark for providing us with the T11A9e hybridomas and J. Singer for a gift of antivimentin rabbit serum. We acknowledge the skillful help of P. Syka. This investigation was supported by Grant 1-R01CA21993 from the National Cancer Institute and by grants from the Hammer Foundation, the Educational Foundation of America, the Pardee Foundation, and the Samuel Roberts Noble Foundation, Inc. The research was conducted in part by the Clayton Foundation, California. R.D. is a Senior Clayton Foundation Investigator.

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