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Cell Biology

Aortic smooth muscle cells contain vimentin instead of desmin

(10-nm filaments/viscera/vasculature/immunofluorescence/two-dimensional gel electrophoresis)

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ABSTRACT **The 10-nm filament subunit proteins of smooth muscle cells in the mammalian aorta were analyzed by immunofluorescence and by two-dimensional gel electrophoresis. Indirect immunofluorescence studies on frozen sections of aorta showed intense staining of the tunica media by antibodies to vimentin, whereas no reaction could be detected with antibodies to desmin. Crude preparations from the tunica media contain a protein with molecular weight and isoelectric point similar to those of vimentin. Desmin was not detected on these same two-dimensional gels. We conclude that aortic smooth muscle cells contain vimentin as the predominant form of 10-nm filament protein subunit, unlike other muscle cells in which desmin predominates. Other immunofluorescence results indicate that the aorta may differ from smaller blood vessels which appear to contain both desmin and vimentin.**

Most cells contain a class of cytoplasmic filaments that have diameters in the range 9–11 nm (1–6). Fibroblasts, leukocytes, endothelium, chondrocytes, and other mesenchymal nonmuscle cells all have vimentin as their predominant 10-nm filament protein (7–11). In contrast, smooth, skeletal, and cardiac muscle have all been thought to contain desmin (also referred to as skeletin) as their predominant 10-nm filament protein (6–8, 12–15). We have investigated the protein composition of 10-nm filaments from visceral and vascular smooth muscle by using immunological and biochemical techniques. We present evidence that the vascular smooth muscle of the aorta has a different 10-nm filament composition than its counterpart in the stomach.

MATERIALS AND METHODS

Cell Culture. Baby hamster kidney fibroblasts, clone 13 (BHK21/C13), and BHK21/C13 cells transformed by the Bryan strain of Rous sarcoma virus (C13/B4) were grown as described (16). Hamster and horse tissues were removed immediately upon sacrifice of the animal and placed on dry ice. Human stomach and portions of aorta free from atheromatous plaques were removed postmortem.

Biochemical Procedures. $NaDodSO_4$ gel electrophoresis was performed according to the procedure of Laemmli (17). The two-dimensional gel electrophoresis procedure has been described (18). Cytoskeletons of tissue culture fibroblasts were prepared according to the method of Brown *et al.* (2) with an extraction buffer (pH 7.4) of 0.5% Triton X-100 in phosphate-buffered saline containing 3 mM KCl. Crude 10-nm filament preparations from various organs were obtained according to the procedure of Lazarides (13, 19). Briefly, the tissue was homogenized in a close-fitting Potter homogenizer and extracted first with 0.5% Triton X-100 in buffered saline and then with 0.6 M potassium iodide/20 mM imidazole, pH 6.8. The residue was solubilized for 12 hr in 20 mM Tris·HCl/8 M urea, pH 7.8. After dialysis against 0.05% $NaDodSO_4$ in water, the samples were concentrated, and mercaptoethanol and glycerol were added to final concentrations of 5% and 10%, respectively.

Antibodies. Vimentin was purified from the cytoskeletons of hamster fibroblasts C13/B4 by preparative $NaDodSO_4$ gel electrophoresis. Desmin was isolated similarly from crude 10-nm filament preparations of hamster stomach. The purified proteins were checked for homogeneity by $NaDodSO_4$/polyacrylamide gel electrophoresis and isoelectric focusing. Two milligrams of vimentin and 2 mg of desmin were injected into a rabbit and a goat, respectively. The injections were given on four separate occasions over a 2-month period. The first two injections contained Freund's complete adjuvant whereas the last two injections contained Freund's incomplete adjuvant. The sera were harvested 2 weeks after the final injections. The antivimentin gamma globulins (anti-V) were purified from rabbit serum by three cycles of precipitation with 33% ammonium sulfate. Antidesmin (anti-D) was prepared similarly from goat serum except that the final concentration of ammonium sulfate was 45%.

Immunoreplica Technique. The immunoreplica technique was performed according to a modification of the procedure described by Showe *et al.* (20). After polyacrylamide gel electrophoresis, the slab gels were overlaid with antiserum mixed 1:1 with a solution of 1.2% agarose in phosphate-buffered saline containing 0.5% Triton X-100. The sandwich was incubated in a moist atmosphere for 6 hr and then the agarose gels were floated off the polyacrylamide gel. After the agarose gels were washed for 4 days in several changes of buffered saline containing 0.25% Triton X-100, they were stained with 0.05% Coomassie blue in 25% 2-propanol/10% acetic acid and destained in the same solution without dye.

Indirect Immunofluorescence. Animals were sacrificed and organs of interest were immediately excised. The specimens were then placed in O.C.T. (Tissue Tek II, Lab-Tek Products, Nagerville, IL) which was then immersed in liquid nitrogen for 1–2 min. Sections 6 μm thick were cut in a cryostat set -12°C and placed on glass slides. The sections were fixed in 1.7% formaldehyde in phosphate-buffered saline, washed in buffered saline containing 0.1 M glycine, made permeable in acetone, cooled to -20°C, and then air dried. For tissue culture specimens, cells were grown on glass coverslips and then processed as described above for the frozen sections (21). For immunofluorescence, the processed slides were incubated with buffered saline containing either anti-V (0.5 mg/ml) or anti-D (0.4 mg/ml) for 1 hr, washed three times in buffered saline for 10 min, and then incubated for 1 hr with the appropriate rhodamine-conjugated second antibody. Tetramethylrhodamine isothiocyanate coupled to goat anti-rabbit IgG or to rabbit anti-goat IgG were obtained from Cappel Laboratories (Cochranville, PA). Blocking experiments were performed by incubating the

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Abbreviations: anti-V, anti-vimentin gamma globulins; anti-D, anti-desmin gamma globulins.

specified gamma globulins with electrophoretically purified desmin (1 mg/ml) or vimentin (1 mg/ml) in buffered saline containing 0.01% $NaDodSO_4$ and 0.5% Triton X-100 for 30 min before application to the specimen. For other control experiments, gamma globulins from preimmune sera were used. The slides were washed again in buffered saline and viewed with a Zeiss standard WL photomicroscope equipped with epifluorescence optics and appropriate filters.

RESULTS

Immunoreplica Technique. We prepared antisera specific for vimentin (anti-V) and for desmin (anti-D). Immunoreplica analysis showed that anti-V reacted specifically with the vimentin (M_r = 55,000; Fig. 1, lane A) in the cytoskeletal preparation of BHK21/C13 cells. Anti-V did not react with the desmin (M_r = 52,000) in the high-salt-insoluble residue of the muscularis externa of hamster stomach which contains very little vimentin (Fig. 1, lane F). Anti-D reacted only with the desmin in a high-salt-insoluble residue of hamster stomach (Fig. 1, lane E). The reaction of anti-D with the BHK21/C13 preparation (Fig. 1, lane C) confirms the previous observation (19, 22), based on biochemical criteria, that these cells contain desmin. Anti-D did not react with the vimentin in this same BHK21/C13 preparation (lane C) or in C13/B4 cells (data not shown) which contain only vimentin and not desmin (19). When preimmune gamma globulins were used, no precipitin bands formed (data not shown).

Immunofluorescence Studies. To confirm the specificity of our antisera, anti-V and anti-D were tested further by immunofluorescent staining of hamster, human, and horse stomach tissue. All species gave the same results. Fig. 2*A* is a frozen section of hamster stomach cut in cross-section and stained with hematoxylin and eosin. When a frozen section of hamster stomach was treated with anti-V, the fibroblasts in the lamina propria and the submucosa stained intensely whereas the muscularis mucosa stained poorly (Fig. 2*B*). This staining was specifically blocked by preincubation of the antiserum with pure vimentin (Fig. 2*D*).

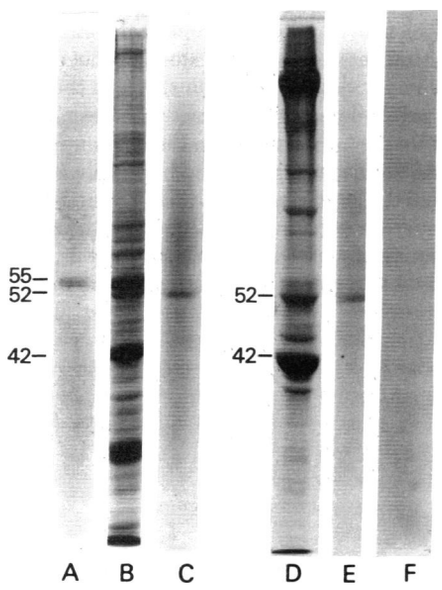

FIG. 1. Specificity of anti-V and anti-D demonstrated by the immunoreplica technique on polyacrylamide gels containing BHK21/C13 cytoskeletal proteins (lanes A–C) and stomach high-salt-insoluble proteins (lanes D–F). Anti-V was used in lanes A and F; anti-D was in lanes C and E. As a guide to molecular weight (shown $\times 10^{-3}$), Coomassie blue-stained polyacrylamide gels, identical to those used for the immunoreplica analysis are in lanes B (BHK21/C13) and D (stomach).

Conversely, the anti-D reacted strongly with the muscularis mucosa, which is a band of smooth muscle, and with isolated cells in the lamina propria (Fig. 2*C*). All staining could be specifically blocked by preincubation of the antisera with pure desmin (Fig. 2*E*). Preimmune controls for both anti-V and anti-D looked similar to the blocked controls in Fig. 2 *D* and *E*. To summarize, anti-D staining was prominent in smooth muscle-rich regions of stomach whereas anti-V staining was confined to the lamina propria and connective tissue regions which are rich in fibroblasts. These data correlate well with the known distribution of vimentin and desmin in the stomach (7, 23).

These same frozen sections of hamster stomach revealed staining of the small blood vessels that course through the submucosa with both anti-D (Fig. 3*B*) and anti-V (Fig. 3*D*). Anti-D stained only the blood vessel and not the surrounding connective tissue, whereas anti-V reacted with both. Most of the anti-D and anti-V staining of the blood vessel wall was on the medial (as opposed to the luminal) side of the internal elastic membrane, which does not stain.

The aorta was examined by immunofluorescence microscopy with these antisera. Fig. 4*A* shows a longitudinal section through a portion of the tunica media (smooth muscle cell layer) of human aorta stained with anti-V. The cells themselves were cut across their short axis. Focal spots of fluorescence are seen to vary in intensity and size. The spots identify the individual smooth muscle cells which are located between the slightly autofluorescent bands of elastin. The nonuniformity of the staining can be predicted by the arrangement of the long and spindle-shaped smooth muscle cells. These cells are oriented circumferentially in the aorta and are offset with respect to one another. Therefore, a certain number of cells are sectioned through the perinuclear region where the diameter is greatest, and other cells are cut closer to their tapered ends. Fig. 4*B* shows the anti-V staining pattern of the aorta cut transversely in which the cells are viewed along their long axis. In this case, the fluorescence was more uniformly distributed because the intermediate filaments run lengthwise along the long axis of the cell. This specific fluorescence was blocked by preincubation of the anti-V with pure vimentin (Fig. 4*C*) but not with pure desmin (Fig. 4*D*). Preimmune controls were negative and similar in appearance to Fig. 4*C*. Staining a section of human aorta with anti-D (Fig. 4*E*) showed no increase of fluorescence above that obtained with preimmune serum. Our anti-D was quite sensitive as evidenced by its strong reaction with the muscularis mucosa in Fig. 2*C*. Similar results were obtained on hamster and horse aortae.

Two-Dimensional Gel Analysis of Aortic Cytoskeletal Proteins. The immunofluorescence data indicated that vimentin and not desmin was the predominant 10-nm filament protein subunit of the smooth muscle cells in the aorta. To characterize the cytoskeletal proteins of the tunica media further, crude 10-nm filament preparations from human aorta were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in the first dimension and polyacrylamide gel isolectric focusing in the second dimension. Human aorta was used because the tunica media can be dissected away easily from the adventitia and intima in which large numbers of vimentin-containing cells are found (10). BHK21/C13 cells, which contain both desmin and vimentin, were used as the standard. In the BHK21/C13 preparation (Fig. 5 *Left*), the 55,000-dalton bands representing vimentin resolved into two major and one minor species with pI in the range 5.50–5.45. The 52,000-dalton region (desmin) resolved into several species, one having a pI approximately equal to that of β-actin. The actin in the 42,000-dalton region focused into α and β species with pI of 5.80 and 5.85, respectively. The

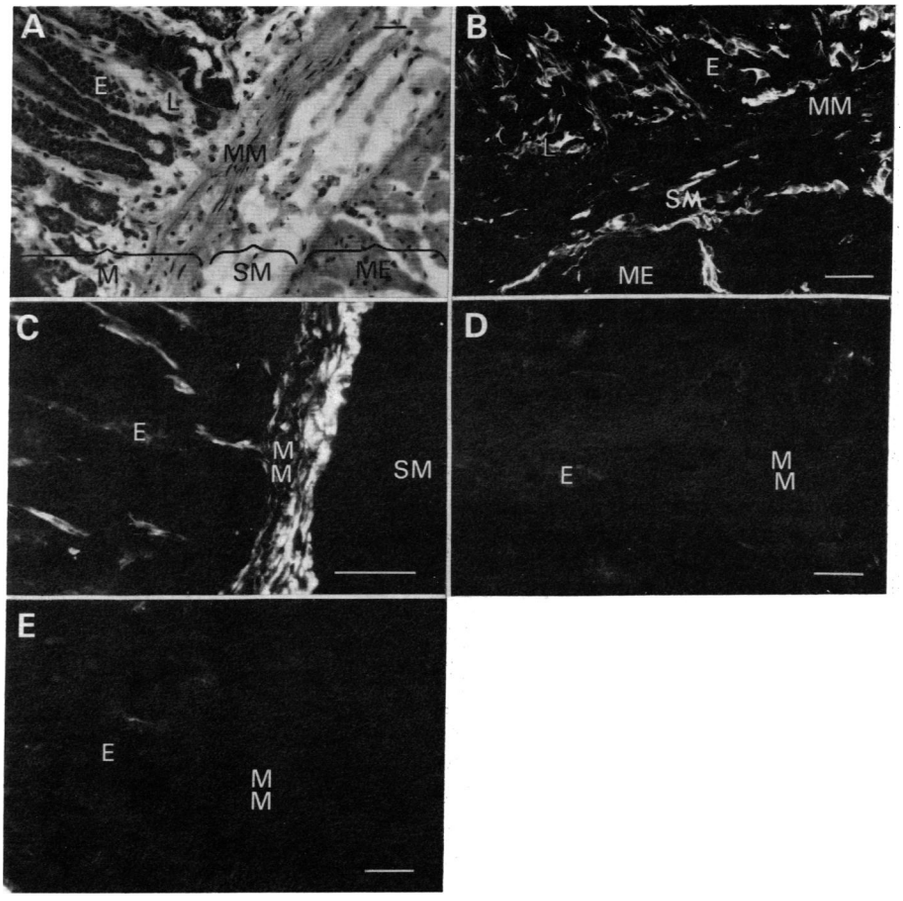

FIG. 2. Frozen sections of hamster stomach. (*A*) Cross section stained with hematoxylin and eosin. (*B–E*) Indirect immunofluorescence with various antibodies. (5-sec exposure through ×40 objective.) (*B*) Anti-V. (*C*) Anti-D; the muscularis externa, which does react with anti-D is not shown here. (*D*) Anti-V preincubated with pure vimentin (1 mg/ml). (*E*) Anti-D preincubated with pure desmin (1 mg/ml). M, mucosa; E, epithelial or glandular cells; L, lamina propria; MM, muscularis mucosa; SM, submucosa; ME, muscularis externa. (Bar = 30 μm.)

55,000-dalton band from human aortic smooth muscle (Fig. 5 *Right*) produced a pattern similar to that of the vimentin from the BHK21/C13. It resolved into several closely migrating species with pI also in the range 5.50–5.45. The actin species included an α-like component and a small amount of a γ-like component, both of which have been described in similar proportions in bovine aortic smooth muscle (24). Moreover, there were no bands in the 52,000-dalton region of the aortic smooth muscle that corresponded to desmin. We conclude that the 55,000-dalton protein in the aorta is, in fact, vimentin and that aortic smooth muscle contains little, if any, desmin.

DISCUSSION

We have shown by the immunoreplica technique and by the immunofluorescent staining of the stomach that our antisera are highly specific for the antigen against which they were made. Moreover, anti-V does not crossreact with desmin as shown by the inability of the antisera to stain the desmin-rich muscularis mucosa of the stomach (Fig. 2*B*) and by its failure to precipitate the desmin in the stomach extract (Fig. 1, lane F). Immunofluorescence microscopy of the tunica media of the aorta with anti-V shows staining of the great majority of the cells, most of which are smooth muscle (25). The staining pattern is too extensive

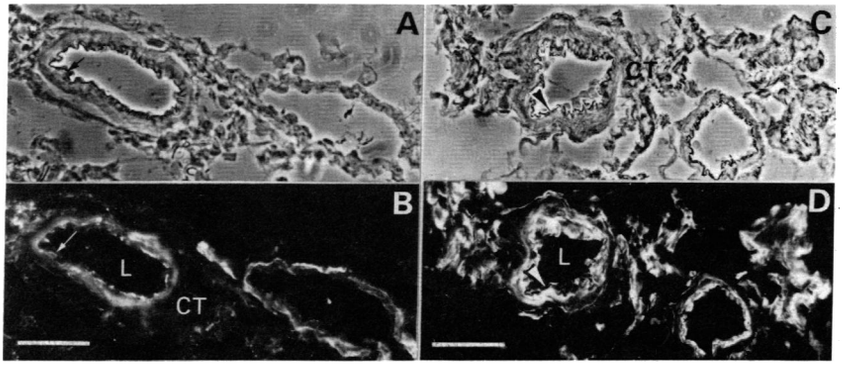

FIG. 3. Submuscosal blood vessels of hamster stomach cut in cross section and stained with either anti-D (*B*) or anti-V (*D*) and viewed by immunofluorescence microscopy. *A* and *C* are phase-contrast images of *B* and *D*, respectively. The strongly fluorescent structure between the two blood vessels in *B* is not readily identifiable. Arrows indicate edge of internal elastic membrane. L, lumen; CT, connective tissue. (Bar = 30 μm.)

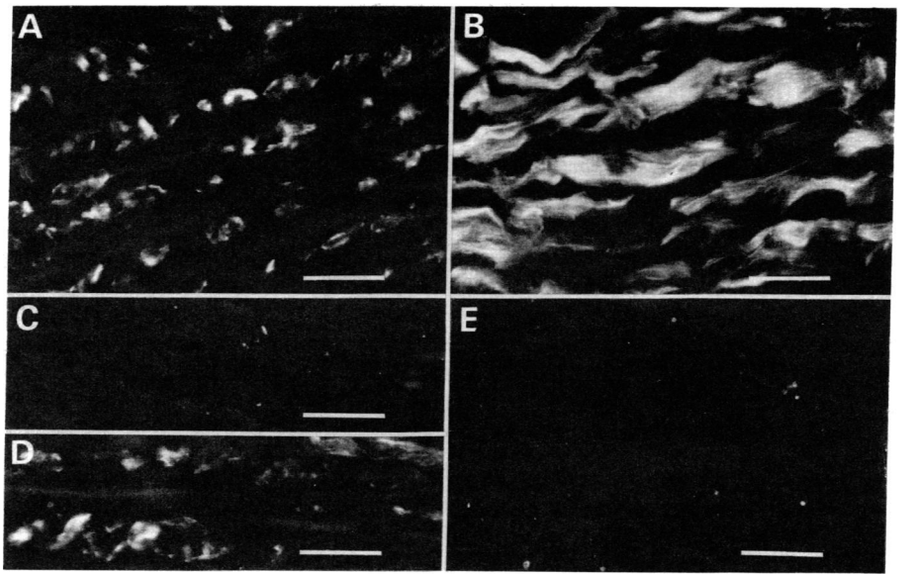

FIG. 4. Immunofluorescence microscopy of frozen sections of human aorta cut either longitudinally (*A*, *C*, and *D*), in which case cells are cut across their short axis or transversely (*B*), in which case cells are cut along their long axis. (*A*) Anti-V; (*B*) anti-V; (*C*) anti-V preincubated with pure vimentin (1 mg/ml); (*D*) anti-V preincubated with pure desmin (1 mg/ml); (*E*) anti-D. (Bar = 20 μm.)

to have resulted from a few fibroblasts or blood cells which may be found in the tunica media. This staining can be blocked by preincubation with pure vimentin but not with pure desmin. Consistent with the immunofluorescent studies, biochemical techniques demonstrate the presence of a molecule in the tunica media of aorta that is similar to vimentin in molecular weight and pI. Because desmin is not detectable by gel electrophoresis or by immunofluorescence, we conclude that vimentin is the predominant 10-nm filament protein of aortic vascular smooth muscle cells. Visceral smooth muscle (Fig. 2*C*), on the other hand, is similar to cardiac and skeletal muscle in that it contains predominantly desmin [although recent reports state that adult skeletal muscle also contains vimentin (refs. 26 and 27, see also refs. 28 and 29)].

Our observations are in agreement with a recent report (30) that the aorta contains vimentin predominantly if not exclusively. However, contrary to that same report, we do find desmin in certain blood vessels. As shown in Fig. 3, the submucosal blood vessels of hamster stomach can be stained with both anti-D and anti-V. The presence of vimentin is not unexpected in light of our findings with the aorta. Yet, unlike the aorta, in the walls of these smaller vessels the staining by anti-D indicates the presence of desmin-containing cells, most likely smooth muscle. Schmid *et al.* (7) reported staining of blood vessels in chicken liver with anti-desmin and anti-vimentin antibodies.

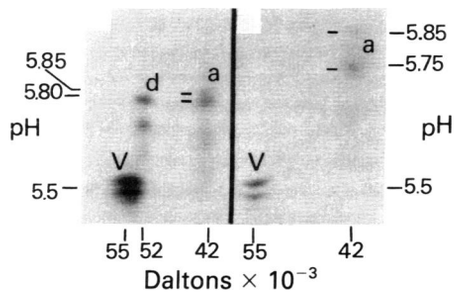

FIG. 5. Two-dimensional gel analysis of cytoskeletal proteins in 40,000- to 60,000-dalton range from BHK21/C13 cells (*Left*) and from tunica media of human aorta (*Right*). Lines indicate actin species present. V, vimentin; d, desmin; a, actin.

The tunica media of rabbit portal vein also appears to contain both vimentin and desmin (Peter Berner, A. P. Somlyo, and A. V. Somlyo, personal communication). Smaller blood vessels are responsible for redistribution of blood flow and therefore must be able to contract *in vivo* as do visceral smooth, cardiac, and skeletal muscle (31). Interestingly, they each contain desmin. In contrast, the aorta undergoes little isotonic shortening *in vivo*, serving mainly as a conducting vessel. Its smooth muscle lacks desmin and instead contains vimentin. Further work is required to see if the presence of desmin is related to *in vivo* contractility.

Note Added in Proof. Many of our findings complement those recently reported by Gabbiani *et al.* (32).

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