Immunolocalization of Meta-Vinculin in Human Smooth and Cardiac Muscles

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Abstract. Meta-vinculin, a vinculin-related protein, has been isolated from human uterus smooth muscle. Specific antibodies to meta-vinculin, which distinguish between meta-vinculin and vinculin, were prepared by absorption of anti–meta-vinculin serum on vinculin coupled to nitrocellulose. Meta-vinculin specific antibody demonstrates only smooth and cardiac muscle specificity and is able to cross-react with a small 21-kD fragment of the meta-vinculin polypeptide chain. This antibody does not interact with protease resistant 95-kD core shared by vinculin and meta-vinculin. Meta-vinculin specific antibody was used for the localization of meta-vinculin in smooth and cardiac muscles by the indirect immunofluorescence method. At the light microscopy resolution level it was found that meta-vinculin and vinculin are localized in the same cellular adhesive structures. Meta-vinculin is present in membrane-associated microfilament-bound plaques of smooth muscle, in intercalated discs and costameres of cardiac muscle. In primary culture of smooth muscle cells from human aorta, meta-vinculin and vinculin were found to be present in focal contacts of the cells. During the cultivation of smooth muscle cells, the quantity of meta-vinculin decreased progressively and finally meta-vinculin completely disappeared from the focal contacts. The data show that in smooth and cardiac muscles meta-vinculin could be a structural component of microfilament-membrane attachment sites, defined earlier by the localization of vinculin.

It is now generally accepted that specific interactions between microfilaments and membranes of muscle and nonmuscle cells play an important role in various cellular phenomena (for review see [14, 24]). Several proteins were found to be concentrated at the ends of actin filaments and to play an important role in the attachment of microfilaments to the plasma membrane (14, 24). One of these proteins, vinculin, has a molecular mass of 130,000 D and was shown to be localized in the junctional plaques of all adherens junctions, including cell–cell contacts and cell–matrix adhesions (5, 12, 15, 16, 19, 29, 30, 36). Vinculin was found in focal contacts of cultured cells, zonula adherens of intestinal epithelial cells, intercalated discs of cardiac muscle and myofibril-sarcolemma attachment regions ("costameres") in striated muscles (5, 12, 15, 16, 19, 29, 30, 36). Vinculin was shown to be expressed in many cell types, notably in blood platelets (21, 32).

Recently, a vinculin-related protein with a molecular mass of ~150,000 D has been isolated from chicken gizzard and porcine stomach smooth muscle (11, 17, 35). This protein was termed as meta-vinculin (34). Unlike vinculin, the expression of meta-vinculin is restricted to muscle cells (11, 17, 18, 33). Amino acid analysis, peptide mapping, and two-dimensional gel electrophoretic analysis demonstrated a high degree of structural relatedness between meta-vinculin and vinculin (11, 17, 35). The difference between the molecular masses of vinculin and meta-vinculin, which is ~20,000 D, enables us to distinguish these proteins by immunoblotting (11, 34). An antibody generated to meta-vinculin completely cross-reacted with vinculin, making the immunolocalization of meta-vinculin in different tissues impossible. The results of in vivo and in vitro experiments led us to suggest that vinculin and meta-vinculin do not have a precursor-product relationship and are distinct proteins, arising from separate mRNAs (11, 35). Both meta-vinculin and vinculin have the ability to interact specifically with talin, α-actinin and a 175-kD membrane protein (1, 6, 7, 20, 27, 40, 41).

In this paper we present immunofluorescent localization of meta-vinculin in human smooth and cardiac muscles. We report that at the light microscopy resolution level meta-vinculin has the same location in smooth and cardiac muscles as had been previously demonstrated for vinculin. Meta-vinculin was found in membrane-associated plaques of smooth muscle, in focal contacts of cultured smooth muscle cells, in intercalated discs and costameres of cardiac muscle.

Materials and Methods

Purification of Meta-vinculin and Vinculin from Human Uterus Smooth Muscle

Human uterus smooth muscle vinculin and meta-vinculin were isolated ac-

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cording to modified procedures, described by (10, 11, 35). 300 g of smooth muscle from human uterus were homogenized in a Waring Blendor with 2 l of deionized water containig 0.5 mM phenylmethyl sulfonyl fluoride (PMSF). The supernatant was discarded and the pellet was resuspended in 2 l of buffer containing 20 mM Tris, 1 mM EGTA, 0.5 mM PMSF, pH 9.0. The suspension was stirred for 60 min at 37°C. The centrifugation was repeated and the pellet discarded. The supernatant was saved and the pH was adjusted to 7.2 with acetic acid, after that 1 M $MgCl_2$ was added to make 10 mM, which stimulated F-actin precipitation. After centrifugation (10 min at 12,000 rpm in JA-14 rotor, Beckman Instruments, Inc. Berkeley, CA) the supernatant was ammonium sulfate fractionated by the addition of, firstly, 14.9 g $(NH_4)_2SO_4$/100 ml and then after centrifugation 6.8 g $(NH_4)_2SO_4$/100 ml more were added to the supernatant. The precipitate was collected, dissolved and dialyzed against a buffer containing 20 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 5 mM Tris-HCl, pH 7.6 (Buffer A). After dialysis the proteins were fractionated on a column of DE-52 cellulose (2.6 × 22 cm) equilibrated in Buffer A. Five major peaks of optical density were eluted from the column. The first one corresponded to vinculin (∼70% pure). The second peak was enriched in meta-vinculin (50-60% pure) as established by SDS-gel electrophoresis. The pooled vinculin and meta-vinculin fractions were dialyzed aganst Buffer A and then were applied to 0.9 × 10 cm columns with hydroxyapatite resin (Bio-Rad Laboratories, Cambridge, MA). Bound proteins were eluted from column with a linear gradient from 0 to 0.2 M K-phosphate, in Buffer A, pH 7.6. Fractions containing meta-vinculin and vinculin were pooled, concentrated and stored at −70°C.

Production and Characterization of Antibody

Antibody to human uterus smooth muscle vinculin was purified from rabbit anti-vinculin serum by affinity chromatography and characterized as described previously (18).

Antibody to human uterus meta-vinculin was obtained by immunization of BALB/*c* mice with 0.1 mg of highly purified meta-vinculin (more than 99% pure) in complete Freund's adjuvant intraperitoneally. 4 w later, the mice were boosted with 0.05 mg of meta-vinculin, and then after another 2 w the mice were boosted again with 0.05 mg of the antigen (two times). The mouse serum was collected and stored in aliquots at −20°C. The absorption of anti-meta-vinculin antiserum on nitrocellulose strips coupled to electrophoretically transferred homogenous human uterus vinculin was done according to (37). The nitrocellulose strip (14 × 1.5 cm) containing ∼1 mg of vinculin was incubated during 2 h with 1.5 ml of anti-meta-vinculin antiserum and this procedure was repeated until the antiserum was unable to interact with vinculin. The last step was to purify antibody to meta-vinculin from mouse serum by affinity chromatography.

In Western blots of proteins from human uterus smooth muscle, the affinity-purified anti-meta-vinculin antibody reacted only with meta-vinculin.

Immunofluorescence Labeling

Freshly isolated human uterus myometrium and cardiac muscle were dropped into freezing isopentane. 3-4 μm longitudinal and transverse cryostat sections were cut at ∼−15°C. Sections were mounted onto glass slides. All sections were incubated in PBS for 5 min before staining at room temperature. For indirect immunofluorescence labeling the sections were incubated with primary affinity-purified anti-meta-vinculin and anti-vinculin IgG's (0.02 mg/ml) for 60 min at 37°C. Sections were washed in PBS over 30 min and then fluorescein-conjugated goat anti-rabbit IgG (diluted 1:40) and rhodamine-conjugated goat anti-mouse IgG (diluted 1:40) were added. After a further incubation for 45 min with secondary antibodies the sections were washed and mounted in PBS with 50% (wt/vol) glycerol. The stained tissues were examined on a Zeiss epifluorescent photomicroscope III with 40× and 100× objectives. Photographs were taken with Kodak Tri-X film. Controls were performed in which the primary antibodies were replaced by: (*a*) preimmune serum (dilution 1:10); (*b*) antibodies to meta-vinculin and vinculin that had been absorbed in advance by an excess of their respective antigens coupled to nitrocellulose.

Cell Culture

Smooth muscle cells from tunica media of human aorta were isolated by collagenase-elastase digestion (8), and cultured in Dulbecco's modified Eagle's medium (DME) (Flow) supplemented with 10 mM Hepes, 6.6 mg/ml sodium pyruvate, 50 μg/ml ascorbic acid, 0.6 mg/ml L-glutamine, 50 μg/ml gentamicin sulfate and 10% heat-inactivated human serum.

Other Methods

To perform SDS-PAGE, the buffer of Laemmli (22) was used. Immunoblotting analysis was done according to the procedure of Towbin et al. (39). Electrotransfer was carried out for 3 h at 400 mA current. The blots were stained by indirect immunoperoxidase technique. The first antibody was mouse anti-meta-vinculin IgG. The secondary antibody was horseradish peroxidase-conjugated rabbit (anti-mouse IgG) IgG. 4-Chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) was used as a substrate for peroxidase. The blue colour developed after 2 min of substrate addition. Two-dimensional electrophoresis was done as described by O'Farrell (28) with slight modifications. Ampholytes used in the lysis buffer and IEF gels were composed of 0.27% Ampholine pH range 3.5-10 (LKB Instruments, Inc.), 1.6% Ampholine pH range 5-7 and 0.8% Ampholine pH range 5-8.

Results

Purification and Properties of Meta-Vinculin from Human Uterus Smooth Muscle

Purification of human uterus smooth muscle meta-vinculin was done according to the modified protocols developed for meta-vinculin isolation from chicken gizzard smooth muscle (10, 11, 35). After selective extraction from human uterus smooth muscle, meta-vinculin was purified by two steps of chromatography on DE-52 and hydroxyapatite columns, while other methods used for meta-vinculin preparation include three chromatographic steps. Meta-vinculin eluted from hydroxyapatite column was at least 99% pure (Fig. 1 *b*), and was able to cross-react with affinity purified anti-vinculin antibody in a Western blot (Fig. 1 *c*). The final yield was ∼4 mg of meta-vinculin from 300 g wet weight of human uterus.

The analytical sedimentation analysis also demonstrated the high degree of homogeneity of the meta-vinculin preparation; the protein sedimented as one boundary and with a sedimentation coefficient $S_{w,20\ C} = 6.5 \pm 0.5$, high molecular weight aggregates were not revealed in the preparation (not shown).

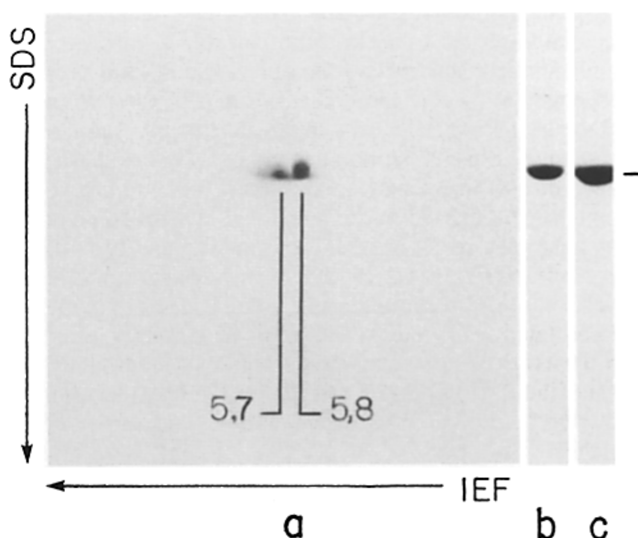

Figure 1. Homogeneity analysis of the human uterus smooth muscle meta-vinculin: (*a*) two-dimensional electrophoresis of purified meta-vinculin; (*b*) SDS-polyacrylamide gel analysis of purified meta-vinculin; (*c*) immunoblot of the same sample as shown in (*b*) with anti-vinculin antibody.

Isoelectrofocusing and two-dimensional gel analysis (Fig. 1 *a*) demonstrate that meta-vinculin has at least two isoforms. The isoform that accounts for 70% of total meta-vinculin amount, has a pI of 5.78. The minor meta-vinculin α-isoform has a pI of 5.70.

Human uterus smooth muscle meta-vinculin isoforms are more acidic than those from chicken gizzard smooth muscle (the α-isoform has a pI of 5.88 and the β-isoform, pI of 6.18) (35). Unlike human, avian smooth muscle meta-vinculin α-isoform has been shown to predominate (35). Two-dimensional gel electrophoresis of porcine stomach meta-vinculin revealed several isoelectric variants which were more basic than those found in human uterus smooth muscle and in chicken gizzard (17). Therefore, meta-vinculin isoform analysis demonstrated that not only avian and mammalian meta-vinculin differ significantly, but also there is a certain variability within the mammalian meta-vinculins too.

Highly purified mammalian meta-vinculin was checked for its ability to bind Triton X-114 detergent according to (4). This was done to probe the presence of hydrophobic domains in the protein. Much like the avian meta-vinculin (35), human uterus meta-vinculin was present in the aqueous phase (not shown), which means that meta-vinculin has no solubility properties typical of integral membrane proteins.

Specificity of Antibody to Meta-Vinculin

For the immunocytochemical and immunoblot analyses described below, antibody specific to meta-vinculin was used. The murine antiserum to human uterus smooth muscle meta-vinculin recognized both meta-vinculin and vinculin (Fig. 2). This antiserum was absorbed six times on strips of nitrocellulose paper coupled to electrophoretically homogeneous human uterus vinculin. The meta-vinculin specific antibody that did not bind to vinculin-nitrocellulose was unable to react with vinculin in immunoblots of meta-vinculin and vinculin preparations (Fig. 2). This antibody was further affinity-purified on meta-vinculin-Sepharose and was used for the detection of meta-vinculin. Fig. 2 *B* demonstrates that affinity-purified anti-meta-vinculin antibody recognized only the 150-kD polypeptide in immunoblots of preparations of muscle proteins from human uterus, aorta, and heart, and did not interact with purified vinculin or any polypeptide from human fibroblast extract; skeletal (sartorius) muscle contained only trace amounts of meta-vinculin (Fig. 2 *C*). To exclude the possibility that antibodies reactive with SDS-sensitive epitopes of vinculin were still present in our preparation of meta-vinculin specific antibody, we used a quantitative immunoassay with anti-meta-vinculin on nondenatured vinculin. We did not find any cross-reactivity of meta-vinculin specific antibody with nondenatured vinculin as well (not shown). Thus, we have obtained meta-vinculin specific antibody suitable for the localization of meta-vinculin in different tissues.

In light of the extreme similarity of vinculin and meta-vinculin, revealed by antibody cross-reactivity (Fig. 2 and reference 11), it was important to study the meta-vinculin domain with which meta-vinculin specific antibody interacted. This was done by the following methods. Meta-vinculin was digested at 37°C by *Staphilococcus aureus* V-8 protease added at 1:100 (wt/wt) to the purified protein. The peptides which had undergone such a digestion were examined by

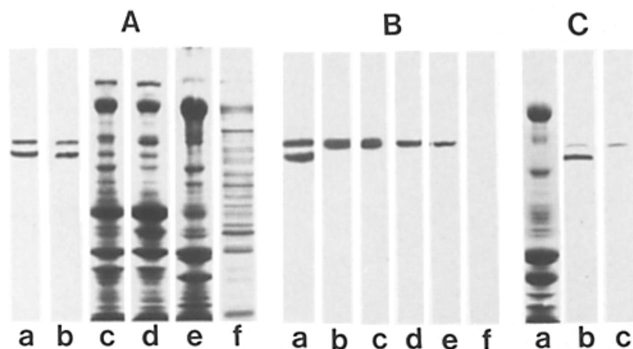

Figure 2. Analysis of specificity of anti-meta-vinculin antibody. (*A*) SDS-polyacrylamide gel analysis of extracts from different human tissues. Proteins were visualized by Coomassie Blue-staining. (Lanes *a* and *b*) Mixture of meta-vinculin (150 kD) and vinculin (130 kD); (lane *c*) uterus; (lane *d*) tunica media from aorta; (lane *e*) cardiac muscle; (lane *f*) foreskin fibroblasts. (*B*) Detection of meta-vinculin in different tissues. Lanes *a-f* show the immunoblots of gels *a-f* from *A*. In *a* nonabsorbed on vinculin-nitrocellulose anti-meta-vinculin antiserum was used; in *b-f* six times absorbed on vinculin-nitrocellulose anti-meta-vinculin affinity purified antibody was used. (*C*) Detection of meta-vinculin and vinculin in human skeletal muscle. (Lane *a*) Coomassie Blue-stained gel of extract from human skeletal muscle; (lane *b*) immunoblot of gel (*a*) with antibody to vinculin; (lane *c*) immunoblot of gel (*a*) with antibody to meta-vinculin.

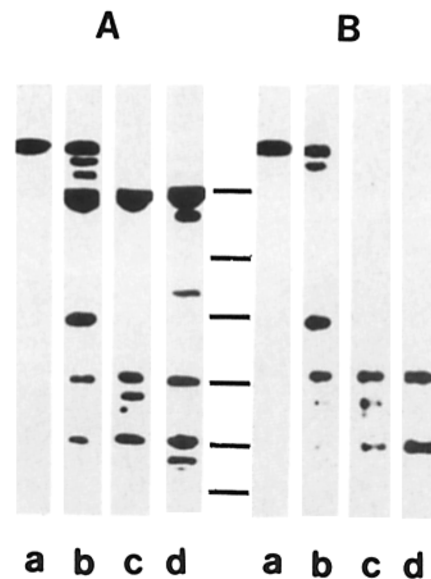

Figure 3. Interaction of meta-vinculin antibody with different meta-vinculin fragments. (*A*) SDS-polyacrylamide gel analysis of the digestion products of meta-vinculin obtained by the action of *S. aureus* V-8 protease. Meta-vinculin was incubated with protease for 0 min (gel *a*), 2 min (gel *b*), 15 min (gel *c*) and 180 min (gel *d*). The reaction was stopped by the addition of hot SDS-gel sample buffer. The position of molecular mass markers (from top to bottom) are indicated by the lines: phosphorilase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20 kD), α-lactalbumin (14.4 kD). (*B*) Immunoblots of the same samples as shown in *A* as obtained using anti-meta-vinculin specific antibody.

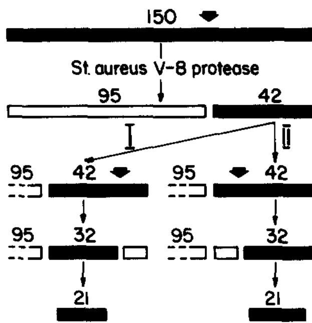

Figure 4. Model for the sequential fragmentation of intact meta-vinculin with *S. aureus* V-8 protease. (*I* and *II*) two possible ways for 42-kD fragment fragmentation. Meta-vinculin-specific antibody reactive fragments are shown as solid bars.

SDS-PAGE and immunoblotting with meta-vinculin specific antibody. Meta-vinculin was degraded first to fragments with approximate sizes of 130, 115, 95, 42, 32, and 21 kD (Fig. 3 *A*). In the course of digestion the 130-, 115-, and 42-kD fragments disappeared from the digestion mixture, as it is seen in 15- and 180-min digest products (Fig. 3 *A*) and new fragments with approximate sizes of 55, 28, and 18 kD appeared. Immunoblotting analysis of the meta-vinculin fragments with the meta-vinculin specific antibody demonstrated that the meta-vinculin specific antibody was able to interact only with small pieces of meta-vinculin with molecular masses of 42, 32, and 21 kD (Fig. 3 *B*), and did not cross-react with the large 95 kD fragment, shared by vinculin and meta-vinculin (11, 17, 25, 35). Fig. 4 summarizes the proposed scheme of intact meta-vinculin fragmentation by *S. aureus* V-8, emphasizing the presence of definite meta-vinculin epitopes on the terminus of the meta-vinculin molecule which are absent from the vinculin molecule. At the present moment it remains unclear from which terminus of the meta-vinculin molecule the fragment binding anti-meta-vinculin specific antibody originates.

Meta-Vinculin Localization in Different Human Muscles

To localize meta-vinculin in muscles, cryostat sections of human uterus, skeletal and cardiac muscles were studied by double indirect immunofluorescent labeling, using affinity-purified antibodies to meta-vinculin (murine IgG) and vinculin (rabbit IgG). In all experiments, immunofluorescence was specific: no staining was observed when anti-meta-vinculin antibody was preabsorbed with meta-vinculin or when nonimmune IgG was used (not shown).

Human Uterus

Indirect immunofluorescence staining of sections of human uterus with antibodies against meta-vinculin and vinculin demonstrated that meta-vinculin was mainly concentrated in

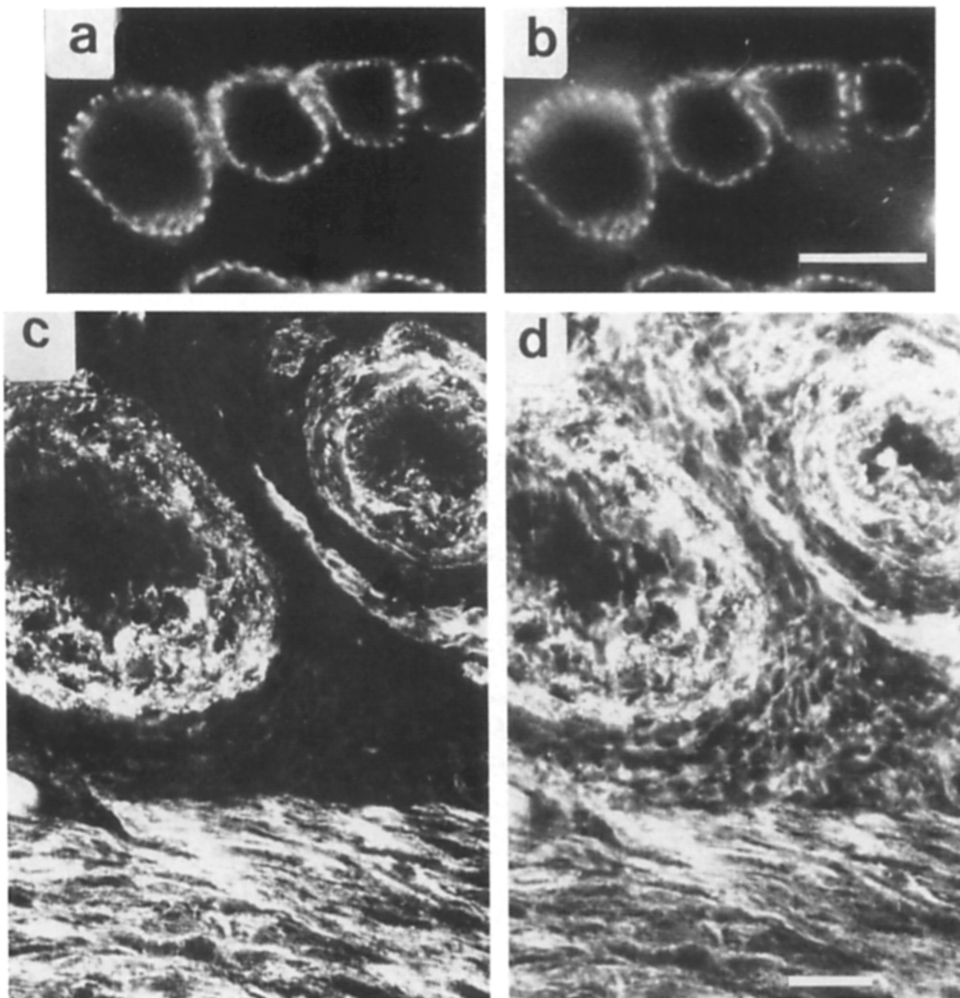

Figure 5. Immunofluorescence localization of meta-vinculin in human uterus. The sections were double labeled with rhodamine-anti-meta-vinculin (*a* and *c*) and fluorescein-anti-vinculin (*b* and *d*). (*c* and *d*) Transverse sections of human uterus smooth muscle cells. Bars: (*a* and *b*) 10 μm; (*c* and *d*) 20 μm.

blood vessels and uterus smooth muscle cells (Fig. 5, *c* and *d*). Antibody to vinculin also cross-reacted with smooth muscle cells, but additionally stained connective tissue cells. Thus, these results confirm the high specificity of the meta-vinculin antibody. In accordance with immunoblotting experiments (Fig. 2) meta-vinculin specific antibody was able to distinguish between smooth muscle cells and fibroblasts of connective tissue. The fluorescence staining of transverse sections of human uterus smooth muscle for meta-vinculin and vinculin exhibited identical dotted labeling along the cell periphery, which corresponds to membrane-bound microfilament-associated dense plaques (Fig. 5, *a* and *b*). No staining was detected intracellularly.

Human Cardiac and Skeletal Muscles

When transverse sections of human cardiac muscles were examined (Fig. 6, *a* and *b*) we observed that fluorescence was confined to cell margins. In addition, there was some staining of structures removed from cell margins, which corresponded to membrane invaginations (30). Occasional bright patches of staining correspond to vessels. The localization of meta-vinculin is very similar to that observed earlier for vinculin (19, 30). It seems that meta-vinculin is concentrated very close to the sarcolemmal membrane. Immunofluorescent analysis of longitudinal sections of cardiac muscle demonstrated two main types of meta-vinculin localization (Fig. 6 *c*): (*a*) meta-vinculin is located in cell–cell contact regions, intercalated discs; (*b*) meta-vinculin's spots are periodically distributed along the cell margins and correspond to the I-bands of sarcomeres around Z-line region. Since there was no difference in the distribution of meta-vinculin and vinculin in the same cardiomyocyte (Fig. 6, *d* and *e*) it is possible to suggest that in cardiac muscle meta-vinculin is a component of myofibril-to-sarcolemmal membrane attachment sites, costameres. Immunofluorescence staining of human skeletal muscle transverse sections did not allow us to localize meta-vinculin in the tissue (Fig. 7). While the anti–vinculin antibody interacted with cell margins (Fig. 7 *b*), anti-meta-vinculin antibody, under conditions of the experiment, practically did not stain the section (Fig. 7 *a*).

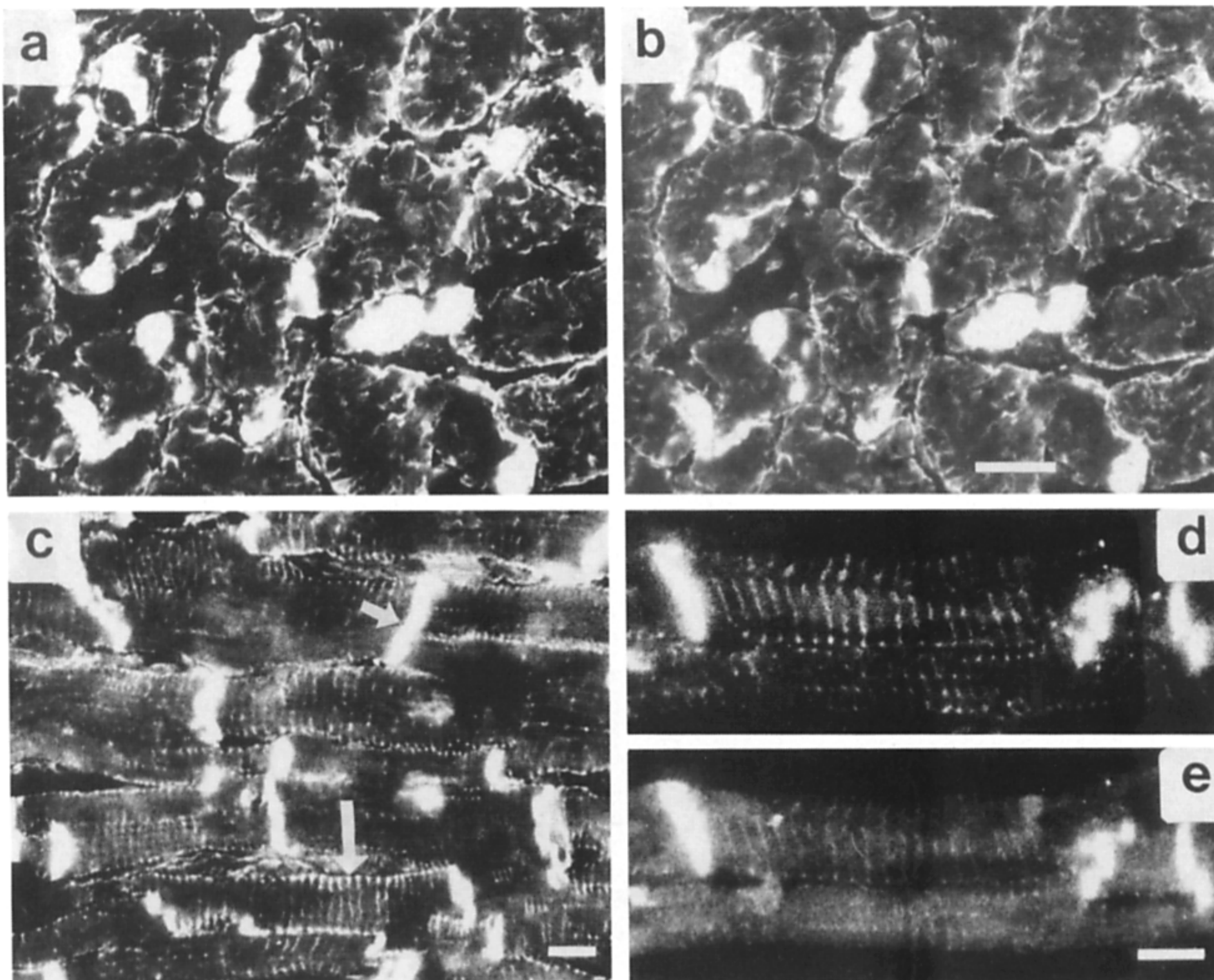

Figure 6. Fluorescence micrographs of meta-vinculin (*a*, *c*, and *d*) and vinculin (*b* and *e*) distribution in human cardiac muscle. Transverse section of cardiac muscle was double labeled with rhodamine-anti–meta-vinculin (*a*) and fluorescein-anti–vinculin (*b*). Longitudinal section of cardiac muscle was labeled with rhodamine-anti–meta-vinculin (*c*) and double labeled with rhodamine-anti–meta-vinculin (*d*) and fluorescein-anti–vinculin (*e*). Meta-vinculin and vinculin are associated with intercalated discs (*small arrow*) and with costameres (*large arrow*). Bars, 10 μm.

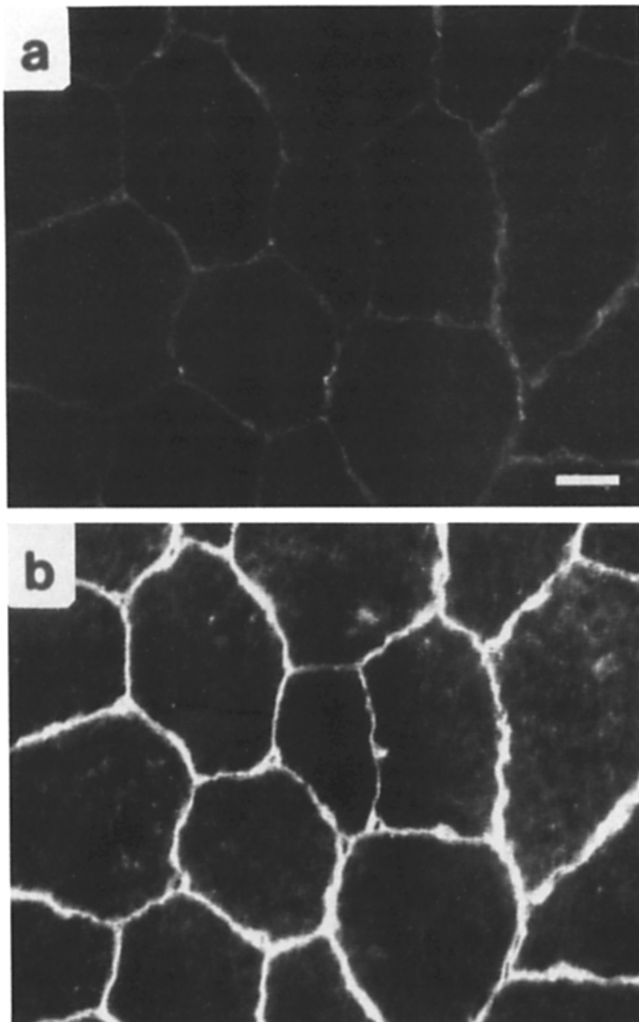

Figure 7. Fluorescence micrographs of meta-vinculin (*a*) and vinculin (*b*) distribution in transverse section of human skeletal muscle. Bar, 20 μm.

Human Aorta Smooth Muscle Cells

Fig. 8 demonstrates the patterns of meta-vinculin and vinculin organization in human aorta smooth muscle cells (primary culture, sixth day). In individual adherent smooth muscle cells (Fig. 8, *a* and *b*) the distribution patterns of meta-vinculin and vinculin were essentially identical. Both proteins were present and had patch-like distribution at sites along the cell periphery. These sites are known to correspond to cell focal contacts (5, 12). Double fluorescent labeling of aorta smooth muscle cell for actin and meta-vinculin indicated that meta-vinculin-rich plaques were associated with termini of F-actin bundles (Fig. 8, *c* and *d*). To determine whether meta-vinculin distribution in smooth muscle cells was altered during cell cultivation, we analyzed meta-vinculin and vinculin localization in primary culture of cells isolated from tunica media of human aorta. These cells did not proliferate up to 10–12 d of cultivation. On the third day of cultivation both meta-vinculin and vinculin were revealed in focal contacts (Fig. 9, *a* and *b*), while by the seventh day in culture meta-vinculin was reduced significantly (Fig. 9, *c* and *d*), and on the fourteenth day, only vinculin could be detected in the focal contacts formed by cultivated smooth muscle cells (Fig. 9, *e* and *f*). It should be mentioned that meta-vinculin was reduced progressively and simultaneously in all the focal contacts.

Discussion

Vinculin is now considered to be a ubiquitous marker of microfilament-membrane association sites (15, 24). Several groups of investigators have demonstrated the existence of vinculin variants (closely related immunologically), but differing in isoelectric point, and in molecular mass (130 kD, vinculin; 150 kD, meta-vinculin). Some of the variants, namely γ-vinculin and meta-vinculin, were found only in muscles (12, 14). The abundance of meta-vinculin relative to vinculin varies depending on the muscle type (2, 11, 17, 33). In vascular smooth muscle, and myometrium the share of meta-vinculin is 40–50%, while in stomach, bladder, and esophagus smooth muscle there is a predominance of meta-vinculin over vinculin (2, 17). In heart muscle meta-vinculin accounts for ~20% of total vinculin (meta-vinculin + vinculin), and only trace amounts of meta-vinculin if any were revealed in human adult skeletal muscle (2, 18). The purpose of this study was to localize meta-vinculin in different muscles using meta-vinculin specific antibody in the indirect immunofluorescence procedure.

Localization of Meta-Vinculin in Cardiac and Smooth Muscles

At the light microscopy resolution level, we have detected meta-vinculin in memrane associated dense plaques of human uterus smooth muscle, in intercalated discs and costameres of cardiac muscle. Under conditions of the experiment we were unable to localize meta-vinculin in human adult skeletal muscle with the immunofluorescence method. According to immunoblotting data the proportion of meta-vinculin in skeletal muscle is rather low, the protein accounts for only 6–8% of vinculin/meta-vinculin total amount (data not shown). The possibility that meta-vinculin detected in skeletal muscle by immunoblotting originates from blood vessels which might be present in the tissue sample cannot be disregarded completely. Therefore, it is still not clear whether adult human skeletal muscles contain any meta-vinculin, or the immunofluorescence method is not sensitive enough to reveal this low abundant protein.

Since smooth and cardiac muscles contain significant amounts of both vinculin and meta-vinculin, the data on vinculin localization in these tissues have to be reevaluated. Meta-vinculin specific antibody enabled us to localize meta-vinculin, while the reaction with an antibody recognizing both vinculin and meta-vinculin can reflect a disposition of either both vinculin and meta-vinculin or meta-vinculin alone. Therefore, since vinculin-specific antibody is not available, vinculin cannot be localized directly in the tissues containing both proteins. However, the direct localization of at least one of the proteins (meta-vinculin) combined with biochemical and immunochemical data led us to propose that vinculin and meta-vinculin colocalize in cardiac and smooth muscles. It should be noted that according to immunofluorescence, immunoelectron microscopy and selective extraction data in muscles, vinculin and meta-vinculin are concentrated in structural elements of the cell (5, 10, 15, 16, 17, 19, 23,

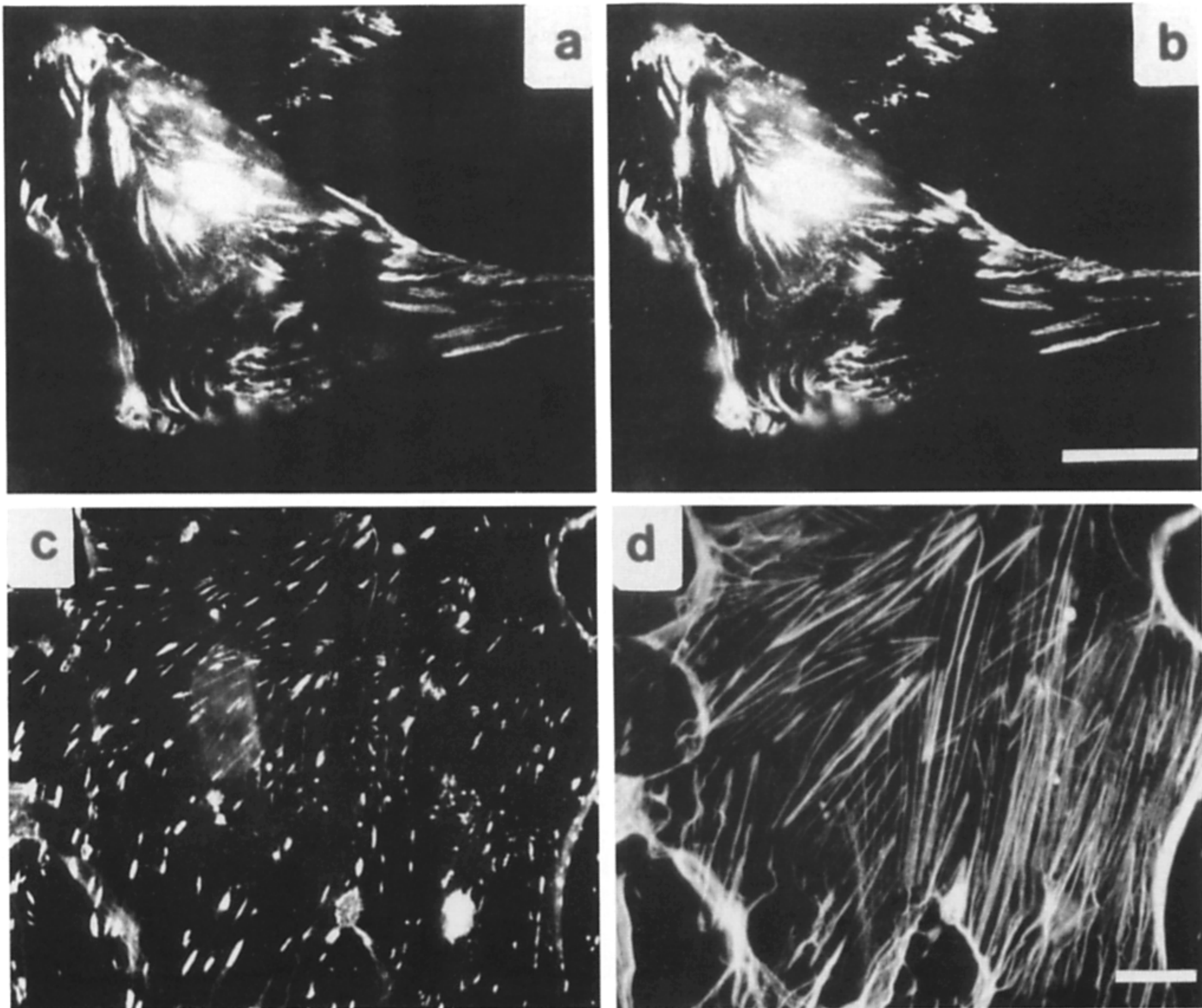

Figure 8. Fluorescence microphotographs of meta-vinculin, vinculin and actin distribution in cultured human aorta smooth muscle cells. Cells were double labeled with rhodamine-anti-meta-vinculin (*a*) and with fluorescein-anti-vinculin (*b*); with fluorescein-anti-meta-vinculin (*c*) and rhodamine-phalloidin (*d*). Bars, 10 μm.

29, 30, 33, 34, 36, 38). Also, in smooth muscle, antibody to vinculin/meta-vinculin similar to meta-vinculin specific antibody interacts exclusively with the antigen localized in membrane-associated dense plaques (15, 34, 36), in cardiac muscle, in intercalated discs and costameres (16, 19, 30, 38). Immunoblotting analysis has revealed vinculin in isolated fascia adherens of intercalated disc membrane (23). In skeletal human adult muscle, which contains a very low proportion of meta-vinculin if any, vinculin was found in costameres. Furthermore, microinjection of fluorescently labeled meta-vinculin and vinculin into cultured myocytes suggests that vinculin and meta-vinculin have the same location in the costameres of developing skeletal muscle cells (33). Thus, it seems unlikely that costameres in the cardiac muscle are devoid of vinculin.

Localization of Meta-Vinculin in Cultured Smooth Muscle Cells

Since meta-vinculin is restricted to muscles, among highly differentiated cells containing the protein smooth muscle cells are the only type that can be easily cultivated for a prolonged period of time. In the smooth muscle, vinculin is located in membrane-associated dense plaques (15, 16, 36), while in cultivated cells it appears to be involved in cell–substrate focal contact formation (12, 24). Double immunofluorescence staining of human aorta smooth muscle cells (primary culture) with: (*a*) anti-meta-vinculin and anti-vinculin antibodies, and (*b*) anti-meta-vinculin antibody and rhodamine-phalloidin demonstrated that meta-vinculin and vinculin were both present in focal contacts. Microinjection of fluorescently labeled meta-vinculin into fibroblasts done by another group also showed codistribution of vinculin and meta-vinculin in focal contacts (33).

Immunochemical studies have shown that meta-vinculin is progressively reduced in human vascular smooth muscle cells in the course of primary culture and can hardly be detected by the onset of intensive cell division (18). In subcultured smooth muscle cells very little meta-vinculin, if any,

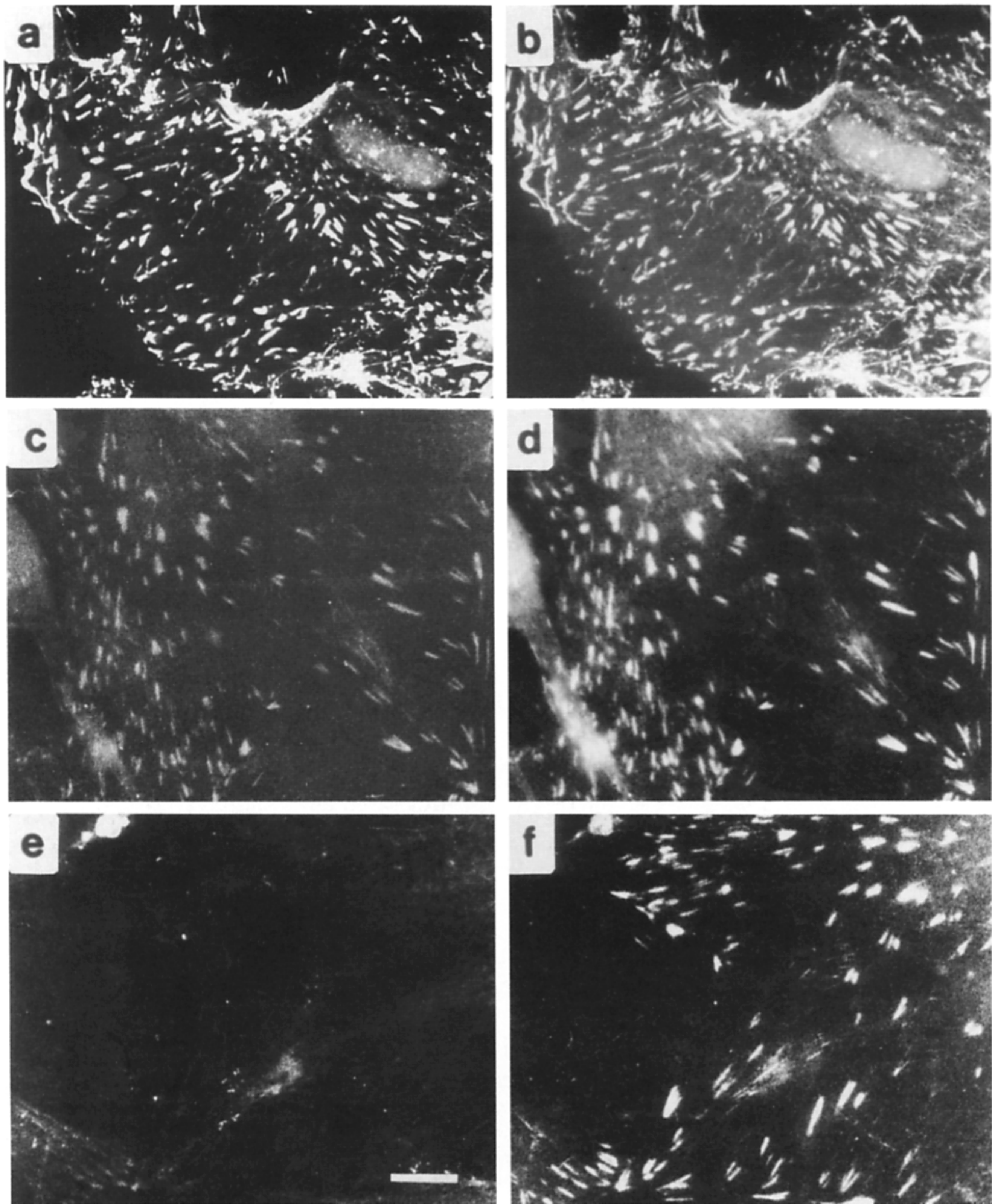

Figure 9. Disappearance of meta-vinculin from focal contacts of primary huan aorta smooth muscle cells. Cells were double labeled with fluorescein-anti-vinculin (*b*, *d*, and *f*) and rhodamine-anti-meta-vinculin (*a*, *c*, and *e*). The cells were cultured during 3 d (*a* and *b*), 7 d (*c* and *d*) and 14 d (*e* and *f*). Bar, 10 μm.

was revealed (18). Meta-vinculin specific antibody enabled us to follow this process in an immunomorphological study: when the cells just finished spreading, meta-vinculin was present in all the focal contacts revealed by anti-vinculin antibodies, whilst in the course of primary culture it disappeared from all the focal contacts simultaneously. Smooth

muscle cells are known to undergo phenotype modulation (8) when grown in culture, and meta-vinculin, like γ-isoform of vinculin (2), can serve as a marker of the "contractile" smooth muscle cell. Therefore, the mechanisms regulating molecular diversity of vinculin in development, in pathological state, as well as during adaptation to culture conditions have to be studied. Now, since vinculin c-DNA from non-muscle source has been cloned and several vinculin c-DNA clones are available (3, 9, 30), they can be applied to investigate the expression of vinculin/meta-vinculin gene(s) in muscle cells.

Taken together, our observations suggest that at the light microscopy resolution level meta-vinculin and vinculin colocalize in smooth and cardiac muscles, and in cultivated smooth muscle cells. It seems that there is no subcellular sorting of vinculin and meta-vinculin in different muscles.

The reason for the presence of two very similar proteins in the junctional structures of smooth and cardiac muscles remains obscure. Vinculin and meta-vinculin have the same solubility, characteristic tail and head structures revealed by electron microscopy, and the same ability to interact with talin and α-actinin (1, 10, 25, 26, 35). However, it should be mentioned that in vivo avian smooth muscle meta-vinculin is more phosphorylated than vinculin (35) but the significance of this fact is poorly understood. Immunofluorescence investigation did not allow us to determine the relative spatial arrangements of vinculin and meta-vinculin in vivo. The possibility should be taken into account that one of the proteins might be concentrated at a closer proximity to the plasma membrane and be directly involved in the linkage of actin filaments to the cell membrane. Immunoelectron microscopy studies could help to clarify this question.

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