

Angiopathic consequences of saturating the plasma scavenger system for actin

(filaments/microthrombi/vitamin D/gelsolin)

J. G. HADDAD*[†], K. D. HARPER*, M. GUOTH*, G. G. PIETRA*[‡], AND J. W. SANGER[§]

Departments of *Medicine, [‡]Pathology and Laboratory Medicine, and [§]Anatomy, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

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ABSTRACT Two plasma proteins, vitamin D-binding protein (actin monomer sequesterant) and gelsolin (actin polymer severing), have been found in association with actin in plasma from ill humans and during experimental injury. *In vitro*, these are the only plasma proteins that display a high affinity for actin. We infused increasing amounts of globular actin intravenously to rats to evaluate its disposition in plasma and tissues. Intravascular filament formation, microthrombi, and endothelial injury were observed, especially in the pulmonary circulation. These pathological changes were not observed when the globular actin in the infusate had been preincubated with the vitamin D-binding protein *in vitro*. Complexes of actin with both proteins were found in the plasma, suggesting a saturable, plasma actin-binding system *in vivo*. Our findings suggest that *in vivo* saturation of these proteins' actin-binding capacities may serve as a paradigm for pulmonary vascular disorders seen during widespread tissue trauma and cell lysis.

Actin, as the major protein of the microfilament system, plays important roles in cell structure and motility. Many intracellular substances are recognized to influence the monomeric or globular (G-actin) and the polymeric or fibrous (F-actin) forms of the protein. At physiological salt concentrations, the formation of F-actin is favored, although the intracellular balance of actin moieties is thought to be regulated by various actin-binding proteins that can influence its polymerization and depolymerization (1–5).

Plasma salt concentrations also favor actin filament formation, and plasma actin-binding proteins are recognized to influence actin behavior, leading to the hypothesis that an actin-scavenger system exists (6–11). The plasma vitamin D-binding protein (DBP), or Gc-globulin, is a 58-kDa polypeptide that exhibits high-affinity ($K_d = 10^{-9}$ M) and high-capacity (5×10^{-6} M) binding of actin monomers (12, 13). Another protein, gelsolin, has been identified in plasma and tissues (11, 14–16). Gelsolin can sever actin filaments, and bind two or three molecules of G-actin (17, 18). The complementary features of monomer sequestration and filament severing may, therefore, constitute an effective mechanism whereby actin can be inhibited from polymerization as well as depolymerized in the circulation. In human (19–22) and experimental (23) conditions characterized by extensive tissue injury, actin-DBP and actin-gelsolin complexes have been found in plasma. The intravenous injection of small amounts of actin into animals results in its association with these two plasma proteins (24–26), further suggesting their roles in a putative mechanism for protection against the formation, growth, and perseverance of circulating microfilaments. In the present studies, we tested the hypothesis that a plasma actin-scavenger system would protect animals from

increasing amounts of infused G-actin until the actin-binding capacity was saturated.

MATERIALS AND METHODS

Materials. Rabbit skeletal muscle G-actin was prepared in 0.01 M Tris/0.2 mM CaCl₂/0.5 mM sodium ATP, pH 7.5 (G-actin buffer) (27). After gel filtration on a 1.1 × 60 cm column of Ultra-Gel AcA44 (LKB) equilibrated in the same buffer, the G-actin was concentrated on Amicon membranes to provide solutions containing the protein at 3–9.33 mg/ml (28). The actin solutions were used within 10 hr of their preparation. Preparations of ¹²⁵I-labeled G-actin (¹²⁵I-G-actin) (25, 29) and fluorescent G-actin (30) were also utilized. The radiolabeled and fluorescent actin preparations were shown to polymerize and sediment with unlabeled G-actin in the presence of 100 mM KCl and 2 mM MgCl₂, as well as bind to purified DBP. Reference murine plasma gelsolin and rabbit antibody to murine gelsolin were kindly provided by J. A. Cooper (Washington University School of Medicine, Saint Louis). Fluorescein isothiocyanate-phalloidin was purchased from Molecular Probes.

Animal Studies. Intravenous infusions of 1–1.5 ml of the G-actin buffer were delivered into the inferior vena cava of 150- to 250-g Wistar rats under barbiturate anesthesia. After a 1- to 2-min infusion, blood was obtained by cardiac puncture or from the abdominal aorta and placed in heparin or heparin-EDTA at 4°C. Tissues were immediately fixed *in situ* and prepared for light, fluorescent, and electron microscopic histological examinations (see legends to Figs. 2 and 3). In some experiments, we coinjected ¹²⁵I-G-actin (25, 29) or rhodamine-labeled G-actin (30) with the unlabeled G-actin to provide additional markers for plasma and histological analyses. Infusions of the buffer alone or buffer containing 40 mg of albumin were not associated with untoward effects or histopathology in the rats.

Analyses. Plasma samples were layered onto 4.8-ml, 5–20% sucrose gradients prepared in the buffers indicated in the legend to Fig. 1. The gradient tubes were centrifuged at 4°C for 18 hr at 40,000 rpm on a SW 50.1 rotor in a Beckman L5-50 B ultracentrifuge. Bottom punctures were made to collect fractions that were analyzed for radioactivity, absorbancy at 280 nm, or immunoreactivity.

Enzyme-linked immunosorbent assays for rat DBP (31) and rat gelsolin were carried out with rabbit anti-rat DBP antiserum (25) and the rabbit anti-murine gelsolin antiserum that was found to cross-react with rat gelsolin (32). Microtiter plates were coated with reference proteins or sucrose gradient aliquots prior to the addition of rabbit anti-rat DBP or rabbit anti-gelsolin antiserum diluted in 1% gelatin/Tris-buffered saline, pH 7.5. Horseradish peroxidase-labeled goat

anti-rabbit antibody (Bio-Rad) was added and color development was effected with *o*-phenylenediamine in citrate buffer with H₂O₂. Wells were analyzed in a Bio-Rad spectrophotometer at 450 nm.

RESULTS

After infusions of 3, 5, or 7 mg of G-actin, no untoward effects were observed in these rats. Plasma samples revealed all of the G-actin to be complexed to DBP, with no evidence of G-actin alone or actin oligomers (Fig. 1 *A* and *B*). After 10- or 14-mg doses of actin, however, a faster-sedimenting shoulder of labeled actin and heavier forms appeared in the plasma (Fig. 1 *C* and *D*). Centrifuged plasma from rats receiving the higher dose of actin revealed filaments that stained clearly with fluorescein isothiocyanate-phalloidin, an agent known to stain F-actin. Infusions of the 14-mg doses led to sudden cardiopulmonary arrest and right heart dilation.

Microscopic examination of the lungs revealed striking abnormalities of the pulmonary arterioles and capillaries (Figs. 2 and 3). Light microscopic autoradiography showed

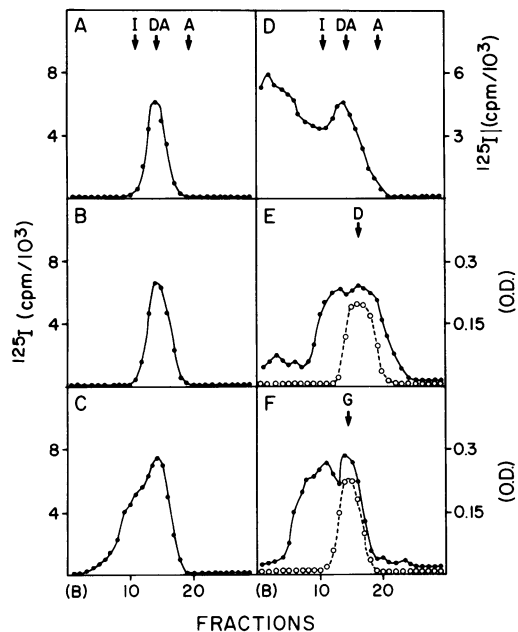


FIG. 1. Sucrose gradient ultracentrifugation of rat plasma samples. Five to 20% linear sucrose gradients were prepared in 0.01 M sodium phosphate/0.15 M sodium chloride/1 mM CaCl₂, pH 7.5. Heparinized plasma was prepared from abdominal aortic blood obtained 1 min after initiating the infusions of G-actin. Sedimentation of reference markers is indicated by the vertical arrows: I, 7S immunoglobulin; DA, 1:1 DBP-G-actin complex; A, G-actin monomers analyzed on gradients prepared in G-actin buffer; D, human DBP; G, murine gelsolin. Analyses of radioactivity distribution are shown in plasmas from rats receiving 5 mg (*A*), 7 mg (*B*), and 10 mg (*C*) of G-actin and ¹²⁵I-G-actin. The dominant sedimentation in these gradients is identical to that of the DBP-G actin complex. After the 10-mg dose (*C*), however, additional faster-sedimenting material is seen, similar to that seen with gelsolin G-actin complexes. The profile observed after a 14-mg dose of G-actin (*D*) reveals additional faster-sedimenting moieties, including material at the bottom of the gradient. Bottom fractions of this gradient revealed filaments on staining with fluorescein isothiocyanate-phalloidin. Other analyses were carried out on gradients layered with plasma from rats receiving the 14-mg G-actin dose. Enzyme-linked immunosorbent assays for rat DBP and rat gelsolin were performed on aliquots of the fractions shown in *E* and *F*, respectively. The sedimentations of rat DBP in normal rat plasma (○) and in plasma from a rat receiving 14 mg of G-actin (●) are shown in *E*. The sedimentation of immunoreactive gelsolin in normal rat plasma (○) and in plasma from a rat receiving 14 mg of G-actin (●) is shown in *F*.

clusters of developed silver grains in the lumina of pulmonary arterioles of rats receiving the 14-mg dose along with ¹²⁵I-G-actin (Fig. 2*A*). At higher magnification, platelet microthrombi (Fig. 2*B*) enmeshed in a dense network of F-actin bundles (Fig. 2*C*) were seen in pulmonary arteries and, occasionally, in alveolar capillaries. Swelling of endothelial cells and interstitial edema were present (Fig. 2*D*). Sections of heart, spleen, and kidney, on the other hand, rarely exhibited F-actin bundles in the lumina of small arteries and capillaries.

Fluorescent micrographs of frozen sections from rats that had been injected with the 14-mg dose of actin containing rhodamine-labeled G-actin confirmed the autoradiographic and ultrastructural observations. Rarely was the injected rhodamine actin found to have polymerized in the capillaries of cardiac tissue (Fig. 3*A* and *B*) or renal tissue. In the larger arterial vessels of the lung some of the injected actin was in a fibrous form (Fig. 3*C* and *D*), and in the alveolar capillary beds there was a high concentration of injected rhodamine actin in a polymerized form (Fig. 3*E* and *F*).

Plasma from rats receiving the saturating doses of G-actin revealed no "free" actin, but faster-sedimenting forms were observed (Fig. 1 *C* and *D*) along with faster-sedimenting forms of DBP (Fig. 1*E*) and gelsolin (Fig. 1*F*) (31, 32). Compared to normal rat plasma, less of these proteins was found in fractions characteristic of their normal sedimentation, and more was found in eluates, suggesting their binding to actin as well as their entrapment in assembled actin forms (Fig. 1 *E* and *F*). Immediate chelation of calcium in blood samples and inclusion of chelator in the gradients did not consistently alter the patterns of sedimentation observed for gelsolin. Its position in these gradients was compatible with one or more bound actin monomers as well as association with assembled actin.

We added G-actin to rat plasma containing labeled rat DBP *in vitro* and showed complete displacement of the rat DBP to the 5–6S region on gradients, when greater than equimolar amounts of G-actin were used (not shown). We also examined the ability of DBP, after preincubation with G-actin *in vitro* to prevent the *in vivo* effects of the highest dose of G-actin. When 66% of the highest G-actin dose was bound to DBP prior to injection, rats survived the infusion, plasma actin polymers were not seen, and intravascular filaments and thrombi were not observed in tissue sections. Thus, by extending the actin-scavenger system *in vitro*, the deleterious effects of actin polymerization and the subsequent microangiopathy were prevented.

DISCUSSION

Our observations appear to demonstrate directly that a plasma actin-scavenger mechanism exists *in vivo* and that its saturation leads to intravascular actin polymerization and severe hemodynamic and microangiopathic consequences. Since DBP-actin complexes can be demonstrated in the blood of patients with fulminant hepatic necrosis and trophoblastic emboli, as well as animals with experimental lung injuries (19–23), it is possible that the plasma actin-binding proteins are protective in a wide variety of circumstances during which rapid and/or extensive cell lysis occurs. Our study's design, including only G-actin, is rudimentary, since actin oligomers, F-actin, and many intracellular actin-binding proteins would be expected to be liberated from lysed cells (1–5). Pulmonary microangiopathy is a frequent occurrence in very ill patients, especially during extensive trauma, shock, and sepsis (34–36). The lungs were the first microvascular bed encountered in our experiments. Whether platelet aggregates formed in the systemic venous or pulmonary arterial system is not known. The mechanisms of endothelial injury and pulmonary edema are not understood but may have resulted

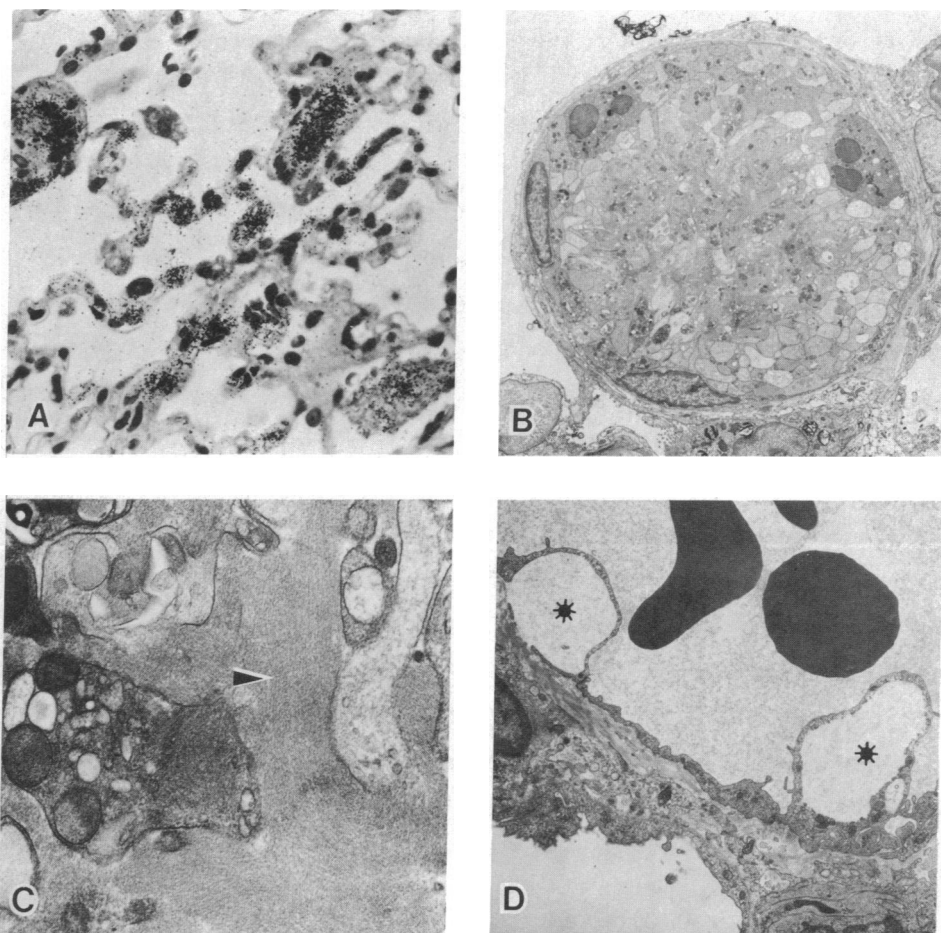


FIG. 2. (A) Representative light microscopic autoradiograms of the lung parenchyma in a rat injected intravenously with 14 mg of G-actin containing ^{125}I -G-actin. Pulmonary arterioles and capillaries contain clusters of developed silver grains, preferentially located over the microthrombi occluding the arterioles. The lung was embedded in paraffin by conventional methods, and 5- μm -thick deparaffinated sections were coated with L-5 photographic emulsion (Ilford) as published (33). After 4 weeks' exposure at 4°C, the sections were developed in full-strength D-19 (Eastman Kodak) and stained with hematoxylin/eosin. ($\times 400$.) (B) Low-power electron micrograph of an intraacinar pulmonary arteriole from a rat injected intravenously with 14 mg of G-actin. The animal died spontaneously of acute heart failure. The lungs were immediately fixed by intratracheal instillation of 2 ml of 1.5% paraformaldehyde/1.5% glutaraldehyde/0.01% tannic acid in 0.1 M sodium-cacodylate buffer (pH 7.2) and processed for electron microscopy. The lumen of the blood vessel is completely occluded by a thrombus composed of platelets and a few neutrophils. ($\times 2000$.) (C) Higher magnification of the thrombus shown in B reveals dense masses of microfilaments (arrowhead) embedding partially degranulated platelets. ($\times 15,000$.) (D) Electron microphotograph of a precapillary muscular lamina by vacuoles (asterisk). No platelets, microthrombi, or microfilaments are seen in the arterial lumen. ($\times 3500$.)

from a direct effect of actin polymerization or indirectly as a consequence of hemodynamic changes and liberation of vasoactive substances from platelets and leukocytes trapped in microthrombi (37–39).

Since the concentration of actin in the cytoplasm is several hundred micromolar, a high-capacity plasma-binding system may be protective in all but the most extensive and rapid occurrences of cell lysis. Impaired circulation, however, would compromise such a system. Together, DBP and gelsolin appear to provide $>10 \mu\text{M}$ capacity to "neutralize" actin in its monomer and polymeric forms. It is of considerable interest that, in studies of $>75,000$ subjects, a deficiency of DBP is unknown, lending support to the concept that deletion of the DBP gene is a lethal mutation. Although binding of vitamin D sterols is the other known activity of DBP, the concentration (12) and clearance (40) of DBP are not apparently influenced by vitamin D supply or metabolism. Recent studies of the DBP genes in man and rat have revealed strong homologies with the albumin and α -fetoprotein genes (41, 42). The ancestral nature and the constitutive expression of the DBP gene may also be related to its product's role in the plasma actin-scavenger mechanism.

Several other studies have reported actin-binding proteins in plasma. In addition to actin antibodies, however, most of these appear to describe depolymerizing or severing (gelsolin or brevin) activities or monomer sequestrants (DBP) or both (10, 15, 43–45). Gelsolin has been identified to be a product of several tissues with a tissue and plasma form, and its activities on F-actin are enhanced in the presence of DBP (13, 46). The actin-binding activity of DBP most closely resembles that of the intracellular protein, profilin, but at 1000 times higher affinity (47, 48).

Minor portions of the plasma DBP and gelsolin appeared in uncomplexed forms in samples that contained polymerized actin (Fig. 1D) that was identified by phalloidin staining. We think that our samples from the left ventricle and abdominal aorta may have reflected an actin occupancy of plasma less pronounced than that likely to have been present in the vena cava, right heart, and pulmonary arteries. Since our studies only included G-actin infusions, they would not faithfully simulate events after cell lysis *in vivo*. Currently available information, however, appears to suggest that our findings identify a protective system (49, 50), operative during pathophysiological conditions.

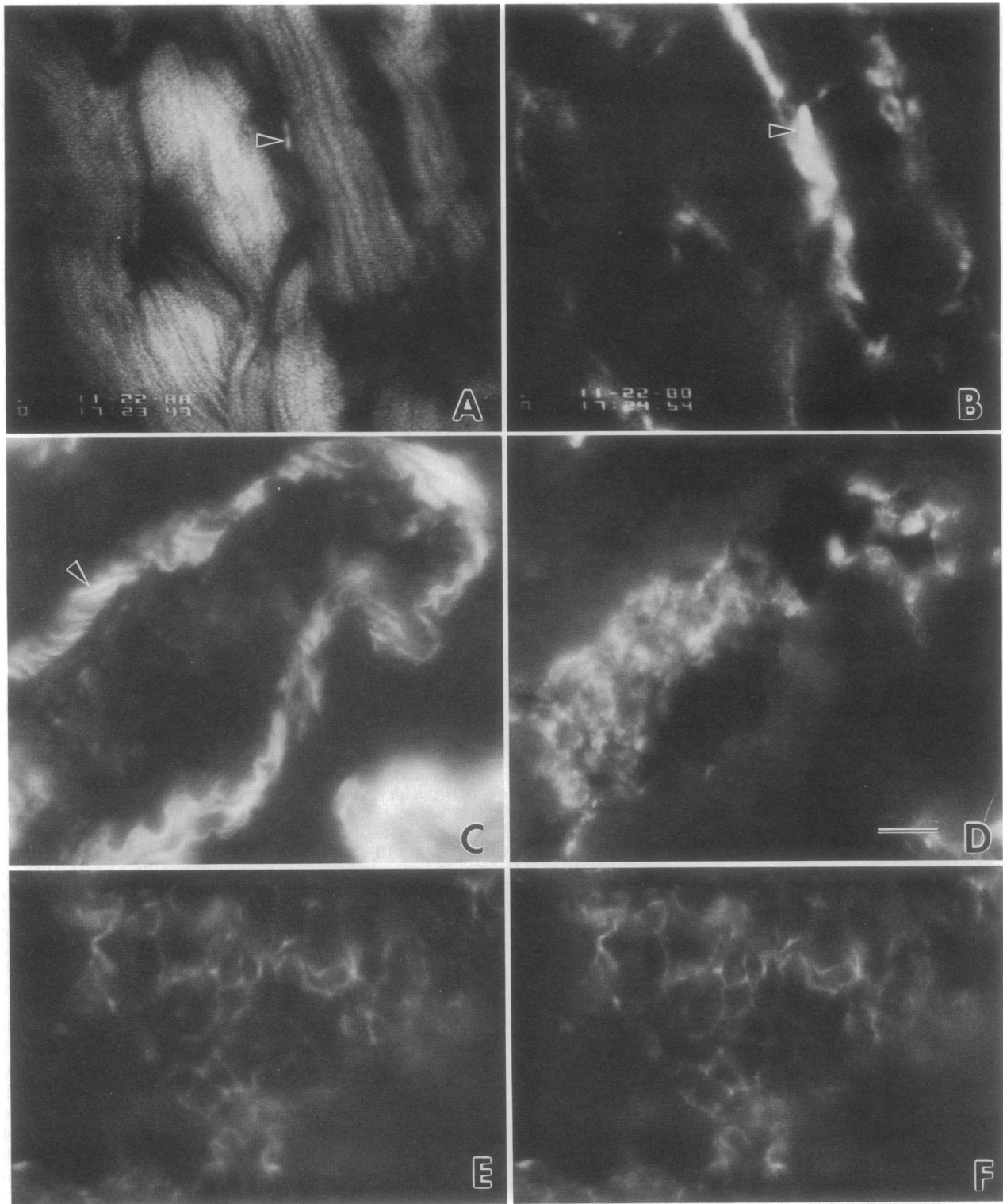


FIG. 3. Sections of heart (*A* and *B*) and lung (*C*–*F*) from a rat that had been injected with 14 mg of G-actin with rhodamine-labeled G-actin as a tracer. (*A*, *C*, and *E*) Sections stained with fluorescein isothiocyanate-phalloidin to localize the distribution of F-actin. (*B*, *D*, and *F*) Same sections viewed to reveal the distribution of the injected rhodamine actin. The arrowheads in *A* and *B* indicate the colocalization of F-actin and injected rhodamine-labeled actin in the capillary bed between two muscle cells. The arrowhead in *C* indicates the smooth muscle lining of an artery in the lung. Note that some of the actin in the arterial lumen is in a filamentous state (*C*). The staining of the aveolar capillary beds with fluorescent phalloidin revealed a colocalization of F-actin (*E*) and the injected rhodamine-labeled actin (*F*). (Bar = 10 μm .)

It is possible that extracellular and intravascular microfilament formation could contribute to angiopathies associated with trauma and inflammation, since vascular injury and platelet activation could lead to microthrombi development. Additional studies, including various actin moieties and/or tissue injuries, should lead to a better definition of the

kinetics and characteristics of the plasma actin-protection system.

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