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Circulating Actin-Gelsolin Complexes Following Oleic Acid-Induced Lung Injury

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Plasma gelsolin is one of two extracellular proteins that bind actin, a major body protein, with high affinity. The authors performed a series of experiments to determine whether tissue injury leads to actin release and the formation of circulating actin–gelsolin complexes. Two functions of plasma gelsolin, filament-nucleating and filament-severing activity, were used to measure total and free gelsolin concentrations, respectively. Both gelsolin and gelsolin–actin complexes nucleate actin assembly, whereas only free gelsolin severs actin filaments. Therefore, nucleation reflects the total gelsolin concentration, severing, the free gelsolin concentration, and the difference, gelsolin–actin complexes. Injection of F-actin in the rat caused a reduction in the free, but not total, gelsolin levels, consistent with the formation of circulating actin–gelsolin complexes. Oleic acid (50 mg/kg) administered intravenously in rats, a treatment that causes acute hemorrhagic pulmonary necrosis, caused the free gelsolin concentration to fall to a greater extent than the total gelsolin concentration, which indicated the presence of circulating actin–gelsolin complexes. Lower doses (9–27 mg/kg) in rabbits caused a qualitatively similar but smaller change in the free gelsolin level. Plasma gelsolin was immunoprecipitated at times when actin–gelsolin complexes were present, as determined functionally, and bound actin was demonstrated by immunoblotting with an anti-actin antiserum. These studies show that considerable amounts of actin are released into the extracellular space during acute lung injury and that circulating actin–gelsolin complexes can be detected in the peripheral blood. (Am J Pathol 1988, 130:261–267)

ACTIN is the major protein within many cell types[1] and because of the mass of muscle in higher organisms, may be the most abundant body protein. Cell injury could therefore release large amounts of actin into the extracellular space, where the ionic conditions favor polymerization of actin into filaments (F-actin), which, in solution, can be many microns in length.

Two plasma proteins bind actin with high affinity: plasma gelsolin and the vitamin D-binding protein (DBP).[2–6] Although each may play a role in the clearance of actin from the circulation,[5,7,8] they differ in how they interact with actin.[9–11]

Plasma gelsolin is a variant of cellular gelsolin, a protein originally isolated from rabbit alveolar macrophages.[12] Plasma gelsolin differs from cellular gelsolin only by an additional 25 amino acid sequence at the amino terminus of the molecule,[13] and both gelsolins are the product of a single gene.[14] Plasma gelsolin has two actin-binding sites and binds with high affinity to either monomeric (G-) or F-actin.[15,16] When plasma gelsolin is added to monomeric actin, it binds the second actin with higher affinity than the first, and thus preferentially forms 2:1 complexes over 1:1 complexes. When added to F-actin, plasma gelsolin severs the filament in a nonproteolytic manner and remains bound to one end of the newly formed filament. If free gelsolin molecules are present, they will sever the filament successively until only 2:1 actin–gelsolin complexes are present, thereby rapidly depolymerizing the filament.[11] Free and complexed (to actin) gelsolin molecules differ in their functional properties. Although free gelsolin can

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sever filaments, actin-gelsolin complexes cannot.[17] Either free gelsolin or actin–gelsolin complexes can accelerate filament assembly from monomers, a property known as nucleating activity.[16,18]

DBP has a single actin binding site and binds to monomeric but not F-actin.[5,6,9] DBP–actin complexes have been reported in the sera of both pregnant women and patients and laboratory animals with fulminant hepatic necrosis.[19–21] Although Thorstensson et al reported, however, the elution of plasma gelsolin (or actin depolymerizing factor) activity and a polypeptide that reacted with an anti-actin antiserum from a DNase I-Sepharose column that had been incubated with human serum, apparently from a single donor,[22] a detailed study of the dynamic formation of gelsolin–actin complexes has not been previously performed.

The different properties of free and complexed gelsolin have made it possible to develop assays to detect actin in plasma after lung injury. Examination of the filament-nucleating and -severing activity of plasma would be expected to provide information about the total concentration of gelsolin as well as the free gelsolin concentration. For example, loss of severing activity (a measure of free gelsolin) without a concomitant loss of nucleating activity (a measure of the total gelsolin content) would indicate the presence of actin–gelsolin complexes in the sample. We describe here experiments in which such assays demonstrated the formation of actin–gelsolin complexes in the peripheral blood of rats and rabbits subjected to acute oleic acid-induced lung injury, with immunologic confirmation that actin was indeed present in circulating complexes.

Materials and Methods

Materials

Male Sprague–Dawley rats were obtained from Charles River Breeders, Wilmington, Massachusetts. New Zealand white rabbits were obtained from Pine Acres Rabbitry, Norton, Massachusetts. Pyrene -iodoacetamide was purchased from Molecular Probes, Eugene, Oregon. A polyvalent antibody prepared to the NH_2-terminal synthetic decapeptide of alpha smooth muscle actin was kindly provided by Drs. Omar Skalli and Giulio Gabbiani, Department of Pathology, University of Geneva. Intravenous cannulas (0.025 inches inside diameter × 0.047 inches outside diameter × 8.0 inches) were prepared from Silastic tubing stock obtained from Dow Corning, Midland, Michigan.

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt[23] and labeled with pyrene-iodoacetamide as described previously.[16] Preparation of monoclonal antibodies to gelsolin has previously been described.[24] Monoclonal anti-gelsolin antibodies were covalently linked to CNBr-activated Sepharose 4B beads (Pharmacia Laboratories, Piscataway, NJ), according to the manufacturer's instructions (1 mg/ml), and were stored in a solution containing 15 mM Tris, 100 mM NaCl, pH 7.4. Protein determinations were performed by the method of Bradford.[25] Serum albumin concentrations were measured by the Bromcresol green binding assay (Sigma Chemical Co., St. Louis, Mo).

Functional Gelsolin Assays

Gelsolin activity was detected in plasma by 1) measuring actin filament nucleating activity (a measure of the total gelsolin concentration) and 2) measuring filament-severing activity (free gelsolin activity) by methods previously described from this laboratory.[26] Briefly, pyrene-labeled G-actin undergoes a 25-fold increase in its fluorescence when appropriate salts are added and the monomers are incorporated into actin filaments (F-actin). The initial rate of polymerization is enhanced by gelsolin and is proportional to the total gelsolin concentration.

Because gelsolin remains bound to one end of an actin filament after severing it, appropriately diluted gelsolin-capped filaments can only depolymerize from the uncapped end. The initial rate of depolymerization under such conditions is proportional to the free gelsolin concentration, because gelsolin-actin complexes cannot sever filaments.

Assay for Plasma-Nucleating Activity

Ten microliters of plasma was added to 140 μl of a solution containing 2 mM Tris, 0.2 mM adenosine triphosphate (ATP), 0.2 mM $CaCl_2$, 0.2 mM mercaptoethanol, 2 mM $MgCl_2$, 150 mM KCl, pH 7.8 (buffer B). Fifty microliters of monomeric pyrene-actin (1.1 mg/ml) was then added, and the change in fluorescence recorded. As described elsewhere,[26] the initial rate of polymerization of actin (d[F]/dt) in the presence of plasma gelsolin under these conditions can be described by the equation:

$$d[F]/dt = k_+ (\text{gelsolin})_{\text{total}}(\text{monomeric actin}) \quad (1)$$

Each day the assay was performed a standard curve was constructed showing the initial rate of polymerization as a function of gelsolin added from a stock solution. The gelsolin activity of samples was determined from triplicate samples.

Assay for Plasma Filament-Severing Activity

Ten microliters of pyrene-labeled F-actin (0.55 mg/ml) was added to 380 μl of buffer B, followed by 5–10 μl of the sample plasma; and the initial rate of depolymerization was determined. The rate of fluorescence decrease is proportional to the rate of actin filament depolymerization, which is related to the number of cuts introduced by free gelsolin by the equation:

$$-d[F]/dt = k_{-} (\text{gelsolin})_{\text{free}} \quad (2)$$

A standard curve relating the initial rate of depolymerization to the amount of free gelsolin added from a known standard was prepared each day, and the filament-severing activity of the sample was measured in triplicate.

Addition of oleic acid to pyrene-actin polymerized in the presence of gelsolin (3 mg/ml) did not alter either the rate of polymerization or the fluorescence of the sample (data not shown), which indicated that the oleic acid injected had no direct effect on the assay of gelsolin activity.

Isolation and Identification of Plasma Actin–Gelsolin Complexes

Sepharose beads linked to monoclonal anti-gelsolin antibodies were used to extract actin–gelsolin complexes from plasma. Fifty-microliter aliquots of a 50% bead suspension were mixed with 100 μl of plasma for 12 hours at 4 C. The beads were washed once in a solution containing 50 mM Tris, 150 mM NaCl, pH 7.4 (TBS), once in TBS containing 0.5 mM $MgCl_2$, and once in TBS containing 2 mM EGTA. Twenty microliters of gel sample buffer (0.1 mM dithiothreitol, 1% sodium dodecyl sulfate, 0.08 M Tris, 60% sucrose) was added to the washed beads prior to electrophoresis on 5–15% SDS-polyacrylamide gels by the method of Laemmli.[27] Separated polypeptides were transferred to nitrocellulose paper, and polypeptides cross-reacting with an anti-actin antiserum were identified by the method of Towbin.[28]

Animal Studies

Adult male Sprague–Dawley rats weighing 300–500 g were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally). Silastic cannulas were implanted in the subclavian vein 2–24 hours before the experiment. The time of implantation did not affect the baseline gelsolin levels of the plasma. The cannulas were flushed with 100 μl of heparinized saline before each use, and the first 200 μl of blood drawn from the cannula was discarded each time. Samples of 0.8 ml of blood were drawn into syringes containing heparin (25 U/ml). Plasma was prepared from the whole blood by centrifugation at 13,000*g* for 5 minutes. Serum was prepared by allowing blood to clot in glass tubes for 2 hours, followed by centrifugation at 13,000*g* for 5 minutes. Oleic acid was suspended in normal saline (800 mg/ml) with 1% albumin and injected via the tail vein (50 mg/kg).[29] Serial samples of blood were obtained from the continuously anesthetized rats at 15–30-minute intervals. The rats were kept anesthetized with additional injections of pentobarbital (30 mg/kg intraperitoneally) as needed for the duration of the experiment.

Blood from New Zealand white rabbits weighing 2.5–4.5 kg was drawn into heparin-containing syringes from the marginal ear veins. The rabbits were continuously anesthetized with intermittent boluses of sodium pentothal, 40 mg/kg intravenously, and closely monitored so that deep anesthesia could be maintained. Oleic acid was administered by ear vein injection in doses of 9–27 mg/kg. Hemorrhagic pulmonary edema was present in each of the animals at the time of death, and a gross inspection of the lungs showed the presence of severe, diffuse hemorrhage, confirming the presence of extensive lung injury.

Results

Effects of Actin on Rat Plasma Gelsolin Activity

The addition of actin to solutions of purified gelsolin or to human serum reduces the filament-severing activity of gelsolin without affecting its nucleating activity.[11,16,17] The observation that gelsolin-actin complexes can nucleate filament assembly as well as free gelsolin indicates that the nucleating activity of a specimen can be used as a measure of the total (free and bound) amount of gelsolin present. The loss of filament-severing activity in such circumstances permits a measurement of the free gelsolin concentration. It would thus be expected that if F-actin were to be released into the plasma *in vivo,* a low level of filament-severing activity (free gelsolin) would be found in the face of a normal level of nucleating activity (total gelsolin).

To determine whether this was the case, the following experiment was performed. First, the plasma gelsolin concentration of a rat was measured (0.23 mg/ml, 2.5 μM). The animal was then given an injection of rabbit skeletal muscle F-actin (8.7 mg/kg, an amount calculated to transiently raise the plasma actin concentration to 5 μM). Plasma was examined before and after the injection for changes in severing and nucleating activity. As shown in Figure 1, the severing activity of the plasma decreased immedi-

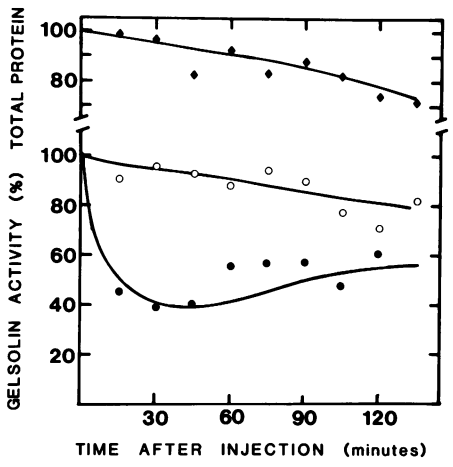

Figure 1—Effect of the injection of F-actin on gelsolin activity of rat plasma. A rat was given F-actin at time zero. The filament severing activity (free gelsolin, *closed circles*), nucleating activity (total gelsolin, *open circles*), and total protein content of the plasma (*diamonds*) were determined. The amount of actin injected was calculated to decrease the free gelsolin concentration by 50%.

ately, whereas the nucleating activity remained constant. Little change in the total plasma protein concentration was noted. Some recovery of plasma severing activity was seen 30 minutes after its nadir. This may represent clearance of gelsolin–actin complexes and/or equilibration of such complexes with the free gelsolin of the extracellular space.

Effect of Oleic Acid Injection on Plasma Gelsolin Activity

Experimental lung injury was induced in rats by administering oleic acid intravenously. Figure 2 shows changes in plasma gelsolin activity in 3 rats given 50 mg/kg of oleic acid. As when purified actin was injected, a loss of filament severing activity (free gelsolin) was seen that is greater than the change in nucleating activity (total gelsolin), consistent with the presence of circulating actin–gelsolin complexes. Similar results were obtained in animals that did not have intravenous catheters placed where blood was drawn either by cardiac puncture or from a tail vein.

Because our monoclonal antibodies to gelsolin do not react with rat plasma gelsolin, and to rule out the possibility that the method used to draw blood was introducing actin into the sample, we injected oleic acid intravenously into rabbits. The results of two experiments are shown in Figure 3. Animals received either 15 mg/kg (panel A) or 23 mg/kg (panel B) intravenously. The free gelsolin concentrations (filament-severing activity) fell to a greater extent than did the total gelsolin concentration, which indicated the presence of gelsolin–actin complexes. Changes in the total protein and albumin concentrations of each sample are shown for comparison. Only small differences between the free and total gelsolin concentrations were seen in 2 animals given 9 and 13 mg/kg of oleic acid.

Immunologic Identification of Actin in Plasma After Lung Injury

The pattern of changes in plasma gelsolin activity in both rats and rabbits given oleic acid were consistent with the hypothesis that actin was released into the plasma after lung injury. Direct demonstration of the presence of circulating gelsolin–actin complexes was performed by immunoprecipitating such complexes. Monoclonal anti-gelsolin antibody conjugated to Sepharose beads was incubated with samples of rabbit plasma. The beads were washed and subjected to elec-

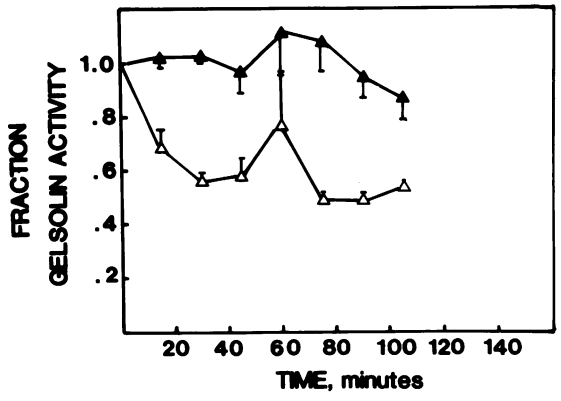

Figure 2—Effect of intravenous oleic acid injection upon the plasma gelsolin activity of rats. Three rats were given 50 mg/kg of an oleic acid suspension. The total (*closed symbols*) and free (*open symbols*) gelsolin levels are shown ± standard deviation.

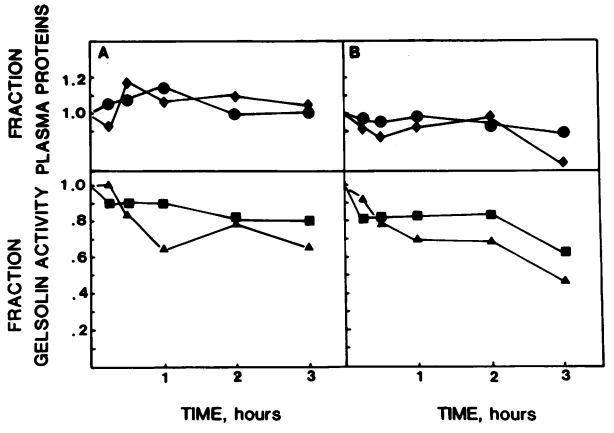

Figure 3—Effect of intravenous oleic acid injection upon the plasma gelsolin activity of rabbits. The rabbit in **A** was injected with 15 mg/kg oleic acid prior to blood sampling, and the rabbit in **B** with 23 mg/kg. Plasma protein concentrations (total protein, *diamonds;* albumin, *circles*) are shown in the upper part, and gelsolin activity (total, *squares;* free, *triangles*) in the lower part of each panel.

trophoresis in sodium dodecylsulfate (SDS)-polyacrylamide gels (SDS-PAGE). The separated polypeptides were transferred to nitrocellulose sheets and incubated with an anti-actin antiserum and a secondary antibody conjugated to peroxidase.

Faint immunoreactivity with a 42-kd polypeptide was variably present in plasma of animals prior to the injection of oleic acid. No single method of blood-drawing consistently caused actin to be present or absent in such uninjured animals. Its variable presence in samples drawn from the same animals within a few minutes makes it uncertain as to whether actin is normally found as a constituent of plasma. After the injection of oleic acid, however, actin was easily detected in plasma. Figure 4 shows changes in plasma gelsolin activity of a rabbit given oleic acid (27 mg/kg), and Figure 5 indicates the results of immunoblotting following immunoprecipitation of gelsolin by anti-gelsolin Sepharose beads. A faint band of immunoreactivity is seen in some samples drawn before oleic acid injection, but distinct bands found in the samples corresponding to the time points shown in Figure 4 indicate release of actin into plasma after the onset of lung injury, in accord with the changes in plasma gelsolin activity.

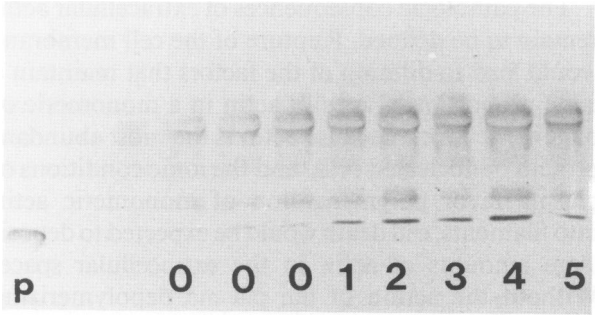

Figure 5—Immunologic detection of actin in plasma of a rabbit with oleic acid-induced lung injury. Plasma samples obtained from the same rabbit shown in Figure 4 were obtained before (*0*) and after (*1–5*) the intravenous injection of oleic acid were incubated with anti-gelsolin Sepharose beads. After washing and electrophoresis of adherent polypeptides on SDS-PAGE, immunoblotting with anti-actin antiserum indicated the presence of anti-gelsolin complexes. Human platelet actin (*P*) was used as a control.

Discussion

The experiments reported here show that changes in plasma gelsolin activity follow the onset of acute oleic acid-induced lung injury in a manner consistent with the appearance of actin–gelsolin complexes in the peripheral blood. The decrease in the filament-severing activity of plasma following the injection of either pure actin or oleic acid indicates either the loss of gelsolin from the circulation or binding of actin to plasma gelsolin, an interaction that renders the gelsolin unable to sever filaments.[11,16,17] Measurement of the second property of gelsolin, the ability to nucleate filament assembly in a calcium-sensitive manner, showed that the fall in free gelsolin concentration detected was in excess of the change in the total gelsolin concentration, and indicated the presence of actin–gelsolin complexes. Confirmation of this conclusion was obtained by demonstrating immunologically that circulating actin–gelsolin complexes could be detected in peripheral blood after oleic acid injection. Because the actin/gelsolin ratio of cells is greater than 30 : 1,[30] it is unlikely that the complexes detected can be accounted for simply by the release of complexes of cellular gelsolin with actin.

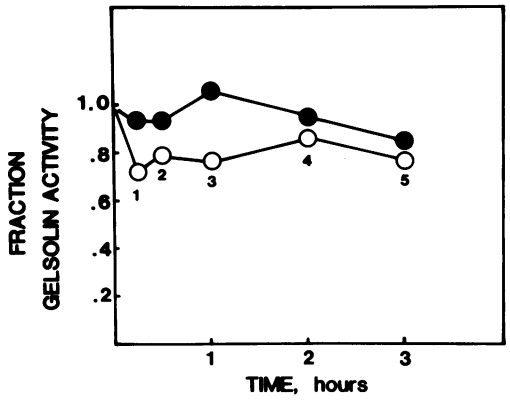

Figure 4—Time course of plasma gelsolin activity following oleic acid injection. A rabbit was given 27 mg/kg oleic acid, and the total (*closed circles*) and free (*open circles*) gelsolin activity of its plasma was determined.

Because DBP is present in plasma at higher concentrations (6–10 μM[31]) than plasma gelsolin (2 μM[32]) and has a higher affinity for monomers than does gelsolin, the demonstration of actin–gelsolin complexes in plasma indicates that plasma gelsolin is exposed to actin filaments as a consequence of cell injury. The relatively high plasma concentration of gelsolin (about 20 mg/dl) suggests that there is an advantage to the organism in having a filament-depolymerizing protein present in the extracellular space. Direct demonstration of this hypothesis has not been possible to date because the large amount of plasma gelsolin (approximately 50% of which is intravascular) present has precluded the injection of sufficient amounts of F-actin into experimental animals to sat-

urate all the plasma gelsolin for an extended period of time. To date, no individuals have been detected who lack plasma gelsolin, though low levels have been found in some disease states (Smith et al, unpublished observations).

The pathologic consequences of extracellular actin remain to be defined. Rupture of the cell membrane would lead to dilution of the factors that maintain a large proportion of a cell's actin in a monomeric or oligomeric form. Because actin is the most abundant protein of nucleated cells, and the ionic conditions of plasma favor polymerization of monomeric actin into filaments, cell death would be expected to deposit large amounts of actin in the extracellular space. Without the action of the plasma depolymerizing proteins (gelsolin and DBP), actin filaments could reach lengths of several microns and might affect blood flow through the microcirculation or cell migration through the extracellular space. The demonstration that long actin filaments can affect fibrin clot formation by inhibiting the lateral association of fibrils into bundles and the abrogation of this effect by shortening the actin filaments with plasma gelsolin[33] give some support for the hypothesis that long actin filaments might interfere with normal physiologic processes.

Anti-actin antibodies (also called anti-smooth muscle antibodies) are known to occur in patients with a variety of diseases.[34-36] This finding suggests that, at the least, actin is presented to immunocompetent cells of the extracellular space under some conditions. Alternatively, extracellular actin may be present in all persons, but only a subset develop antibodies. In either case, these antibodies indicate that extracellular actin is found in other conditions and is not peculiar to the oleic acid injury model.

Study of the formation and clearance of actin–gelsolin complexes in various disease states may be useful in studying tissue injury and inflammation. Specificity of the damage cannot be determined with the techniques reported here, however, because both muscle and nonmuscle actins bind to gelsolin. The use of antibodies specific for different types of actin may provide a way of using these techniques in the study of complex disease states.

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