Monocytes and Macrophages Synthesize and Secrete Thrombospondin

By Eric A. Jaffe, Joseph T. Ruggiero, and Domenick J. Falcone

Thrombospondin, one of the major glycoproteins released from α-granules of thrombin-stimulated platelets, is a disulfide-linked trimer of 160,000-dalton subunits. Cultured human monocytes secreted thrombospondin (determined by an enzyme-linked immunosorbent assay) into the culture medium in a time-dependent manner (1.45 μg/10⁶ cells/24 hr); secretion was totally blocked by cycloheximide (1 μg/ml). ³⁵S-thrombospondin was isolated from ³⁵S-methionine–labeled human monocyte postculture medium with rabbit polyclonal anti-thrombospondin coupled to protein A-Sepharose. The immunosolated ³⁵S-thrombospondin migrated in sodium dodecyl sulfate-polyacrylamide gels after reduction with a molecular weight of 169,000. Similar results were obtained using mouse resident peritoneal macrophages. Elicited peritoneal macrophages harvested from mice pretreated with endotoxin, casein, or thioglyc late secreted much less thrombospondin than did resident macrophages harvested from control mice. Thus, monocytes and macrophages from two different species synthesize and secrete thrombospondin, and the rate of synthesis of thrombospondin appears to depend on the state of activation of the cells.

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MATERIALS AND METHODS

Cells

Human monocytes were isolated from freshly drawn non-anticoagulated blood (40 ml) that had been defibrinated by shaking for ten minutes in a 125-ml Ehrlenmeyer flask containing 25 sterile glass beads (5-mm diameter). ²⁹ The defibrinated blood was diluted 1:1 with medium 199 (M.A. Bioproducts, Walkersville, Md), 40 ml layered over 10 ml of Ficoll-Hypaque (Pharmacia, Piscataway, NJ), and centrifuged at 2,400 g for 20 minutes at 20 °C. The interface containing mononuclear cells was removed, and the cells washed twice in medium 199 by centrifugation at 1,000 g for seven minutes. Monocytes were isolated by incubating the mononuclear cell fraction in T-25 flasks containing medium 199, 2% human serum, penicillin (80 U/ml), streptomycin (80 μg/ml), fungizone (2 μg/ml), and L-glutamine (1.6 mmol/L) for two hours at 37 °C in 5% CO₂. Nonadherent cells were removed by washing the cell layer three times with medium 199; the resulting attached monocytes were cultured in medium 199 containing 20% human serum and the above-mentioned additives until the monocytes were used for experiments. Adherent cells obtained by plating mononuclear cells on glass coverslips were >99% monocytes when examined on Wright-Giemsa–stained preparations; 97.5% contained nonspecific esterase,²¹ and 93% ingested latex particles (density = 1.099 μm; Dow, Indianapolis).

Resident and elicited macrophages were obtained from the peritoneal cavity of Nelson-Collins strain (NCS) female mice (Rockefeller University, New York) as described by Edelson and Cohen.²² Cells were elicited by intraperitoneal injection of 1 ml of either 3% thiglycollate broth (Difco, Detroit) or 1% caseinate or 0.001% endotoxin (lipopolysaccharide B, Escherichia coli 0127:B8, Difco) in phosphate-buffered saline. Four days following injection, elicited macrophages and a resident cell population (from an un.injected cohort) were harvested in Dulbecco's phosphate-buffered saline. Four days following injection, elicited macrophages and a resident cell population (from an un.injected cohort) were harvested in Dulbecco's phosphate-buffered saline and collected by centrifugation (400 g, ten minutes). The cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM), containing 20% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Mouse peritoneal cells (approximately 10 x 10⁶ per flask) were dispensed into T-25 flasks and incubated in 5% CO₂ at 37 °C for two hours. Nonadherent cells were removed by washing, and the resulting monolayers were incubated with DMEM containing 10% FCS, penicillin, and streptomycin until they were radioactively labeled. Radioactively labeled proteins synthesized by human and mouse monocytes were prepared by incubating washed 25-cm² cell monolayers for 24 hours at 37 °C in 4 ml of methionine-free minimal
essential medium containing 20% rabbit serum, Trasylol (60 U/mL), and 25 μCi/mL of 35S-methionine (1,212 Ci/mmol, New England Nuclear, Boston). The radioactive postculture medium was removed, protease inhibitors added as previously described, the medium centrifuged at 8,000 g for two minutes to remove cells and debris, and frozen at -35 °C. In some studies, radioactive extracts of cell layers were prepared as follows: the cells were washed twice, removed with a rubber policeman, pelleted by centrifugation, and dissolved by boiling for five minutes in 2% sodium dodecyl sulfate (SDS) containing protease inhibitors as described. The resulting extract was frozen at -35 °C.

In experiments in which the accumulation of thrombospondin antigen in the postculture medium was measured by enzyme-linked immunosorbent assay (ELISA), monocytes were cultured in minimal essential medium containing 20% rabbit serum in 2-cm² wells of multiwell plates. After three days in culture, the monocytes were washed, incubated for 45 minutes with medium 199-20% rabbit serum, and the medium removed and replaced with fresh medium 199-20% rabbit serum. At various times after the last medium change, the postculture medium was removed, centrifuged at 8,000 g for two minutes, and frozen until assayed. The monocytes were dispersed by incubation with 5 mmol/L EDTA in HEPES-buffered saline for 20 minutes with intermittent agitation and counted in a Coulter (Hialeah, Fla) counter.

**Immunofluorescence Microscopy**

Immunofluorescence microscopy was performed on monolayers of monocytes cultured on glass coverslips. The cells were stained as previously described.

**Preparation of Anti-thrombospondin Antibodies**

Human platelet thrombospondin and polyclonal rabbit (kindly provided by Dr Larry Leung) and monoclonal mouse anti-thrombospondin (kindly provided by Dr Deane Mosher) were prepared as previously described.

**ELISA of Thrombospondin**

Thrombospondin in monocyte postculture medium was measured by specific ELISA using mouse monoclonal anti-thrombospondin, as previously described.

**Isolation and Analysis of Radioactively Labeled Monocyte Proteins**

35S-labeled thrombospondin was isolated from radiolabeled postculture medium and cells by immunoadsorption with rabbit anti-thrombospondin antiserum as described ("immunoisolation"). Immunoisolated labeled proteins were analyzed by polyacrylamide slab gel electrophoresis according to the method of Laemmli, using a 4% acrylamide stacking gel and 7.5% separating gel, followed by autoradiography after treatment with an autoradiography enhancer (ENHANCE, New England Nuclear, Boston). Samples were run both unreduced and reduced with dithiothreitol. Molecular weights were estimated using the following 14C-labeled marker proteins (New England Nuclear): myosin (200,000), phosphorylase a (94,000), bovine serum albumin (68,000), and ovalbumin (45,000) as size markers. Anti-ovalbumin, used as a control, was obtained from Capp Laboratories (Cochranville, Pa).

In experiments in which thrombospondin production by mouse resident peritoneal (control) macrophages and elicited peritoneal macrophages obtained from mice treated with casein, thiglycollate, and endotoxin were compared, thrombospondin was immunoisolated from labeled postculture medium as described above. Total incorporation of 35S-methionine into protein secreted into the postculture medium was measured by precipitation with trichloroacetic acid. Total macrophage cell protein was measured by dissolving buffer-washed monolayers in 0.2 N NaOH and assaying the solubilized material for protein by the Lowry et al procedure.

**RESULTS**

**Quantification of Secretion of Thrombospondin**

Human monocytes were cultured for three days, washed, and placed in fresh culture medium. Thrombospondin in the postculture medium was quantified by ELISA. By 24 hours, the monocytes had secreted 1.45 μg thrombospondin/10⁶ cells, and the level of thrombospondin in the postculture medium was 380 ng/mL. Secretion of thrombospondin by the monocytes was completely blocked by culturing the cells in the presence of cyclohexamide (1 μg/mL). In similar experiments, we have previously demonstrated that human fetal lung fibroblasts and umbilical vein endothelial cells secreted 5.8 and 21.0 μg/10⁶ cells/24 h, respectively.

**Immunofluorescence Studies of Thrombospondin**

In order to characterize the thrombospondin synthesized by human monocytes, human monocytes were cultured in medium containing 35S-methionine for 24 hours. When the labeled postculture medium was incubated with rabbit polyclonal anti-thrombospondin serum coupled to protein A-Sepharose, anti-thrombospondin specifically immunoisolated 35S-methionine-labeled protein with apparent sizes of >400 kd when analyzed without reduction (Fig 1e) and 159 kd when analyzed after reduction (Fig 1k). Purified platelet thrombospondin migrates similarly with and without reduction. No labeled proteins were immunoisolated in control experiments with anti-ovalbumin (Fig 1, f and l). Radioactive bands of similar size were seen in the starting postculture medium (Fig 1, d and j). Similar experiments performed on resident murine peritoneal macrophages yielded identical results (Fig 2).

However, when similar experiments were performed on labeled cell extracts prepared from human monocytes treated with 2% SDS, no 35S-thrombospondin was isolated (Fig 1, a through c and g through i). Immunofluorescence studies performed with the same anti-thrombospondin antiserum on human monocytes cultured for one to seven days did not demonstrate any significant cellular or extracellular immunofluorescence (data not shown).
Effect of Macrophage Activation on Thrombospondin Synthesis

In order to test the effects of macrophage stimulation on thrombospondin synthesis, mice were stimulated by intraperitoneal injection of endotoxin, casein, or thioglycollate and the elicited macrophages harvested along with resident macrophages from untreated mice. The macrophages were placed into culture and labeled with $^{35}$S-methionine for 24 hours. Labeled thrombospondin was immunoisolated from postculture medium as described above. Thrombospondin production by macrophages derived from mice treated with endotoxin, casein, or thioglycollate was markedly depressed (13%, 56%, and 9% of control, respectively) when quantified on a dpm/mg cell protein basis (Table 1). To eliminate the possibility that the reduction in thrombospondin synthesis was due solely to a generalized decrease in protein synthesis, incorporation of $^{35}$S-methionine into trichloroacetic acid-precipitable protein in these samples was also measured. While overall protein synthesis by the elicited macrophages was somewhat decreased (ranging from 66% to 86% of control), thrombospondin synthesis by macrophages derived from mice treated with endotoxin, casein, or thioglycollate was still markedly depressed when expressed as percent of total protein synthesis (19%, 63%, and 14% of control, respectively; Table 1). It is unlikely that the decrease was due to extensive proteolysis of thrombospondin by activated macrophages, because immunoisolates obtained with anti-thrombospondin from the postculture medium of both resident and activated macrophages incubated with $^{35}$S-methionine in the presence of Tranysol contained only two major bands (160 kd and 145 kd) when analyzed by polyacrylamide gel electrophoresis after reduction.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>TSP (dpm/mg Cell Protein)</th>
<th>TCA-Precipitable Protein (dpm/mg Cell Protein)</th>
<th>Percent TSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.39 ± 0.12</td>
<td>7.58 ± 1.57</td>
<td>1.86 ± 0.23</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>0.18 ± 0.03</td>
<td>5.01 ± 0.05</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>Casein</td>
<td>0.78 ± 0.42</td>
<td>6.50 ± 2.97</td>
<td>1.18 ± 0.11</td>
</tr>
<tr>
<td>Thioglycollate</td>
<td>0.13 ± 0.01</td>
<td>4.98 ± 0.25</td>
<td>0.26 ± 0.01</td>
</tr>
</tbody>
</table>

Mice were injected peritoneally with the indicated stimuli four days before sacrifice. Peritoneal macrophages were isolated, cultured, and labeled with $^{35}$S-methionine and labeled thrombospondin (TSP) and labeled total protein quantified as described in Materials and Methods. Data are expressed as mean ± SD for duplicate samples. Three other experiments yielded similar data. TCA, trichloroacetic acid.
DISCUSSION

In the present study, we have shown that human peripheral blood monocytes and murine peritoneal macrophages synthesize and secrete thrombospondin into the extracellular medium. In contrast, we could not demonstrate the presence of thrombospondin either intracellularly or in the form of an extracellular matrix. Human monocytes and murine peritoneal macrophages have both been shown to synthesize fibronectin and secrete it into the extracellular medium. However, like thrombospondin, fibronectin, by immunofluorescence microscopy, was not present in the form of an extracellular matrix and only became visible intracellularly in a small number of cells (25%) after the cells were cultured for at least five days. Similarly, apoprotein E (ApoE), a constituent of lipoproteins (mol wt 33,000) synthesized by murine macrophages, was detected intracellularly by immunofluorescence microscopy in only 11% of murine macrophages at a time when ApoE accounted for 2.5% of the total secreted protein. Since thrombospondin secreted by macrophages (Table 1) accounted for 1.8% of total secreted protein, our inability to demonstrate thrombospondin intracellularly in monocytes is consistent with findings obtained for other monocyte/macrophage proteins.

There are significant and extensive parallels between thrombospondin and fibronectin. Both proteins are multimers of disulfide-linked subunits and are synthesized by multiple cell types, including endothelial cells, fibroblasts, smooth muscle cells, monocyte/macrophages, and, presumably, megakaryocytes, since platelets contain large amounts of thrombospondin and fibronectin in their $\alpha$-granules. Endothelial cells, smooth muscle cells, and fibroblasts secrete and incorporate both fibronectin and thrombospondin into their extracellular matrix in the form of fibrillar meshworks, and thrombospondin and fibronectin codistribute in these meshworks. This codistribution is consistent with the interactions observed between thrombospondin and fibronectin using affinity chromatography. Fibronectin supports cell adhesion, modulates cell morphology, acts as a nonspecific opsonin, binds to native and denatured collagen, thrombospondin, heparin, fibrin, and staphylococci, has lectin activity, binds to human monocytes, and is chemotactic for both fibroblasts and monocytes. Thrombospondin binds to type V collagen, fibronectin, histidine-rich glycoprotein, fibrinogen, and heparin, has lectin-like activity, and is necessary for the second phase of platelet aggregation.

In vivo studies using both skin and rabbit cornea models suggest that fibronectin (as detected by immunofluorescence) is not present in normal tissue, but instead, is deposited along with fibrinogen/fibrin as a result of an acute injury and acts as a temporary extracellular matrix until a more permanent matrix constructed out of collagen, laminin, and other components of the normal extracellular matrix can be formed. Since thrombospondin also binds fibrinogen and collagen, and thrombospondin codistributes with fibronectin in vitro, thrombospondin may also contribute to a provisional matrix following injury. The recent demonstration that human fibroblasts bind and degrade thrombospondin is consistent with this concept.

When murine peritoneal macrophages were activated in vivo, production of thrombospondin as a percentage of protal protein synthesized and secreted into the medium was specifically decreased by 37% to 86%. The degree of activation, as measured by the rise in the number of macrophages elicited from treated mice v control mice, varied from one group of mice to another, though the mice were treated with the same doses of stimuli. Macrophages from animals that were poorly stimulated (ie, small rises in the number of macrophages elicited v control) synthesized only slightly decreased amounts of thrombospondin, whereas macrophages from mice that were strongly stimulated secreted markedly diminished amounts of thrombospondin. These decreases in thrombospondin secretion are consistent with the decreases observed by others for the secretion of fibronectin and ApoE by activated murine macrophages.

Monocytes interact at several points with the coagulation system, since they possess tissue factor, synthesize and activate factor X, bind thrombin and factors VII and IIa, activate prothrombin, and interact with and bind to platelets. The specific role of thrombospondin in monocyte and macrophage function is not yet known, but it may relate to the ability of thrombospondin to enhance cell–cell interactions, as exemplified by its role in platelet–platelet interactions.

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