Activation-dependent Contractility of Rat Hepatic Lipocytes in Culture and In Vivo

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Abstract

Hepatic lipocytes are perisinusoidal cells that have been thought to be analogous to tissue pericytes, a cell type with purported vasoregulatory properties. However, we and others have recently demonstrated that lipocytes acquire markers of smooth muscle cells or myofibroblasts only after liver injury, via a process termed "activation." In this study, we document lipocyte contractility on collagen lattices and examine the importance of activation in this process. In culture, lipocytes became contractile only after spreading and activating, coincident with expression of smooth muscle α actin, a marker of activation (1990. *Virchows Arch. B Cell Pathol.* 59:349). After 5 d in culture, lipocytes induced rapid and sustained contraction of collagen lattices (to 43.7±2.3% of their original size 24 h after detachment). There was no contraction of lattices containing hepatocytes. Scanning electron microscopy demonstrated intimate associations of lipocyte cell membranes and collagen fibrils. Reduction in cell volume during contraction was also prominent. Lattice contraction by lipocytes was proportional to cell number. Serum was a potent stimulator of lipocyte contraction, as were endothelin types 1, 2, and 3; the effect of serum and endothelin 1 were additive. Neither thrombin, angiotensin-II, serotonin, nor the cytokines PDGF and TGFβ induced contraction. Cytochalasin B treatment resulted in concentration-dependent inhibition of contraction.

As a test of the in vivo relevance of the culture findings, lipocytes were isolated from fibrotic animals and examined immediately after adherence. Whereas lipocytes from normal liver were initially compact, smooth muscle α actin negative and noncontractile, cells from animals with hepatic injury due to CCl_4 displayed an activated appearance, expressed smooth muscle α actin, and were contractile immediately after adherence. Additionally, IFN-γ, an agent which blocks lipocyte activation (1992. *Hepatology.* 16:776), inhibited lipocyte contraction. The data document that normal (i.e., quiescent) lipocytes are not contractile, but that activation is associated with the development of contractility. These findings suggest that a role for lipocytes in organ contraction or vasoregulation may be confined to injured, not normal liver. (*J. Clin. Invest.* 1993. 92:1795–1804.) Key words: lipocyte contractility • extracellular matrix • smooth muscle • actin • pericyte

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Introduction

Wound-healing research over the past 30 years has established that actin-containing cells, termed myofibroblasts, populate contractile scars (1). Myofibroblasts have been implicated as mediators of wound contraction in several tissues, including liver (2, 3). Although the origin of myofibroblasts in liver is a continuing subject of debate, mounting evidence suggests that they arise from resident sinusoidal cells known as lipocytes, also termed Ito, fat-storing, or vitamin A-storing cells (4–6). Morphologic studies in injured liver have identified "transitional" cells with vitamin A droplets characteristic of lipocytes that also display prominent thin filaments typical of myofibroblasts (4). In addition, morphometric studies have shown that with progressive liver injury, the number of lipocytes decreases while the number of myofibroblasts increases proportionally, implying an interconversion of the two cell types (7). This phenotypic change from a quiescent lipocyte to a myofibroblast-like cell has been termed "activation" and is an important feature of liver injury. Other characteristics of lipocyte activation in vivo include increased proliferation, enhanced interstitial collagen production (type I > III), and new expression of smooth muscle α actin (8–12).

Cellular features of activation have also been extensively characterized in culture models that recapitulate the in vivo injury response. Normal (quiescent) lipocytes and those in early culture contain abundant retinoid esters and are minimally proliferative and fibrogenic (13–16). With progressive growth on uncoated plastic, lipocytes release retinoids, proliferate, and produce increased amounts of collagens (type I > type III > type IV) and glycosaminoglycans (16–19). Activation is also associated with de novo expression of smooth muscle α actin (9, 10).

Expression of both smooth muscle α actin and the muscle-specific intermediate filament desmin by lipocytes suggests a relationship to smooth muscle cells (20–21). On this basis, it has been proposed that lipocytes function as "liver specific pericytes," a cell type with purported vasoregulatory properties in capillary beds (22–24). Two recent studies have supported this concept, the first demonstrating that rat hepatic lipocytes induced wrinkling of silicone membranes in response to eicosanoids, and the second by showing that passaged human lipocytes respond to several vasoactive mediators with an increase in intracellular free calcium (25, 26). However, these two earlier reports neither quantitated contraction by lipocytes, extended the findings to a model of in vivo liver injury, nor examined the relationship between contractility and activation. In this investigation, we have examined lipocyte contractility on a matrix composed of type I collagen, which predominates in fibrotic liver (27), and have shown that contractility is activation dependent.

Methods

Animal model of hepatic fibrosis. Hepatic fibrosis was induced in retired male breeder Sprague-Dawley rats (400–550 g; Charles River

Breeders, Boston, MA) by carbon tetrachloride (CCl_4) administration (28). Animals were maintained on standard chow and water ad lib. and received humane care. A 1:1 mixture of CCl_4 (Mallinckrodt Chemical Works, St. Louis, MO) and 100% corn oil (Best Foods, Englewood Cliffs, NJ) was administered at a dose of 0.1 ml CCl_4/kg body wt at weekly intervals via gavage. This method reproducibly results in early bridging fibrosis within 3–4 wk, advanced bridging fibrosis by 8 wk, and cirrhosis with portal hypertension at 10–12 wk, as previously described (10, 28). Control animals received corn oil on the same schedule.

Cell isolation and culture. Lipocytes were isolated from retired male breeder Sprague-Dawley rats (450–550 g) as described (29). In brief, after in situ perfusion of the liver with 0.25 mg% pronase and 0.013 mg% collagenase (both from Boehringer Mannheim Biochemicals, Indianapolis, IN), the dispersed cells were fractionated on a discontinuous Stractan (Champion Paper Co., Libby, MT) density gradient. Lipocytes from fibrotic livers were isolated by increasing the concentration of collagenase by 20%. Lipocytes were ≥ 99% pure as assessed by intrinsic vitamin A autofluorescence. After isolation, lipocytes were suspended in modified medium 199 (30) containing 20% serum (10% horse/10% calf; Flow Laboratories, Inc., Naperville, IL). Hepatocytes were isolated from adult male Sprague Dawley rats (250 g) by in situ perfusion of the liver with 0.01 mg% collagenase. Hepatocytes were ≥ 95% pure as assessed by morphologic inspection. Hepatocytes were grown in the same medium containing 5% calf serum. Cells were grown on or in type I collagen lattices (see below) in 24-well flat-bottom culture plates (Corning Glass Works, Corning, NY) or 35-mm culture dishes (Nunc, Inc., Naperville, IL). Lipocytes were plated at a density of 2.5×10^5 cells/ml of culture media, unless otherwise stated. Hepatocytes were plated at 1×10^6 cells/ml of culture media. Plating efficiency was routinely determined by direct cell counting of adherent cells vs. the total number of cells plated. Plating efficiency was 50–75% for lipocytes and 75–80% for hepatocytes. All cells were maintained in a humidified 2% CO_2 incubator at 37°C. For lipocytes, medium was changed 24 h after plating and every 48 h thereafter. For hepatocytes, medium was changed 2 h after plating and then at 24-h intervals.

Rat kidney fibroblasts (University of California, San Francisco, Cell Culture Facility) were grown in Ham's F-12 medium (Gibco Laboratories, Grand Island, NY) and DME (Flow Lab., Irvine, Scotland) at a ratio of 1:1 (Ham's/DME) in 10% FCS. At confluency, cultures were washed twice with PBS (Gibco Laboratories) and incubated with 0.5% trypsin/0.2% EDTA (Boehringer & SoehneGmbH, Mannheim, Germany) for 1 min. Detached cells were resuspended in Ham's/DME with 10% FCS, placed on top of collagen lattices, and maintained in a moisturized incubator at 37°C.

Collagen lattice preparation. Contraction of lipocytes on collagen lattices was performed in 24-well flat-bottom tissue culture plates (Corning Glass Works) or 35-mm plastic culture dishes (Nunc, Inc.) as described (31), with modifications noted below. Culture vessels were preincubated with PBS containing 1% BSA (500 μl per well, 1 ml per 35-mm dish) for at least 1 h at 37°C, then washed twice with PBS and air dried. A combination of 8 parts Vitrogen (Celltrix Corp., Santa Clara, CA), 1 part 10X MEM (Gibco Laboratories), and 1 part 0.2 M Hepes (resulting in a final collagen concentration of 2.4 mg/ml) was mixed at 4°C. The solution was added to the culture vessel and incubated for 1 h at 37°C to allow gelation. Lipocyte or hepatocyte suspensions were layered on top of formed lattices, except as noted in Results. In some experiments, cells were mixed with collagen solution at 4°C and were thus distributed within the lattice. 1 h after gelation, medium was added to lattices.

After the stated period of growth in culture, collagen lattices containing cells were detached by gentle circumferential dislodgement of the lattice using a 200-μl micro-pipette tip. Contraction was monitored as change in lattice diameter (which was used to calculate area) over time.

Rat kidney fibroblasts were assayed in an identical manner to that for lipocytes. Release of collagen lattices containing fibroblasts was undertaken 24 h after plating.

Immunocytochemistry. Cell cultures were washed with PBS (Gibco Laboratories) and fixed with methanol (4°C, 10 min). Specimens were incubated in PBS containing 0.1% powdered dry nonfat milk (Carnation Co., Los Angeles, CA), and 150 mM ammonium acetate and 2% goat serum (both from Sigma Chemical Co., St. Louis, MO) for 30 min, washed, and incubated with anti-desmin (1:100; Dako, Santa Barbara, CA) and anti-smooth muscle α actin (1:200; Sigma Chemical Co.) for 12 h at 4°C in PBS containing 0.1% dry nonfat milk, 15 mM ammonium acetate, and 2% sheep serum. Specimens were washed three times in the same buffer (without serum) and incubated with affinity-purified, fluorescein-linked, anti-mouse IgG, from sheep (Amersham Corp., Arlington Heights, IL) and affinity-purified, Texas red–linked, anti-rabbit IgG, antibody from donkey (Amersham Corp.) for 1 h (each 1:250). Specimens were again washed three times and stored at 4°C. Control specimens were incubated with nonimmune IgG and irrelevant monoclonal antibody.

Cellular quantitation. Lattices were washed twice with PBS and completely digested in Ham's/DME containing 0.015 mg% collagenase (Boehringer Mannheim Biochemicals) over a period of 2 h at 37°C. The released cells were centrifuged (1,500 *g* for 5 min), and sonicated in 2 M NaCl buffered in 0.025 M monobasic sodium phosphate and 0.025 M dibasic sodium phosphate, pH 7.4. DNA content was measured by fluorimetric assay (32).

Smooth muscle α actin immunoblot for fresh cell isolates. Fresh cell isolates were suspended in a solution containing 62.5 mmol/liter Tris-HCl, pH 6.8, containing 1% SDS, 10% glycerol, and 2% 2-mercaptoethanol. After boiling for 5 min, identical amounts of total protein extract as determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA) were separated by SDS-PAGE (8.0% acrylamide) under reducing conditions and transferred to nitrocellulose. Nonspecific binding was blocked by preincubation of the blot in full-strength PBS containing 5% powdered milk (Carnation) and 2% sheep serum (Sigma Chemical Co.) for 1 h. The nitrocellulose was then incubated with monoclonal smooth muscle α actin antibody, diluted 1:2,000 overnight at 4°C. Bound primary antibody was detected with biotinylated sheep anti-mouse antibody (1:4,000), followed by avidin and biotin (each 1:1,250; Amersham Corp.) and reaction with 3-3′diaminobenzidine (Sigma Chemical Co.) followed by 0.1% H_2O_2. Positive control smooth muscle α actin protein was obtained from normal rat aorta, homogenized by Polytron (Brinkmann Instruments, Inc., Westbury, NY), and processed as for lipocytes.

Smooth muscle α actin immunoblot for cell cultures. Individual collagen lattices were digested in Ham's/DME containing 0.015 mg% collagenase (Boehringer Mannheim Biochemicals) over a period of 2 h at 37°C. Equal numbers of suspended cells were centrifuged, resuspended, and separated by SDS-PAGE (8.0% acrylamide) under reducing conditions. Immunoblotting was carried out as described for fresh cell isolates, above.

Vasoactive mediators. Endothelins 1, 2, and 3, 5-hydroxytryptamine (serotonin), and angiotensin II were reconstituted in 50 mM ammonium acetate and stored at −70°C. Bovine thrombin (108 NIH units/mg) was dissolved in NaCl and Tris HCl, pH 7.0 (per manufacturer), and stored at −70°C. All vasoactive mediators, as well as bovine and equine plasma (which was substituted for serum as a contractile mediator in some experiments), were obtained from Sigma Chemical Co.

Endothelin measurement. Endothelin levels in culture medium and serum were measured by RIA using [125-I] endothelin-1 (Amersham, Les Ulis, France) as described (34).

Cytokines (IFN-γ, TGFβ, and PDGF). IFN-γ (Amgen Corp., Thousand Oaks, CA) and PDGF BB homodimer (PDGF Inc., Boston, MA) were reconstituted in PBS (Gibco Laboratories) containing 2 mg/ml BSA and stored at −70°C. TGFβ-1 from porcine platelets (R & D Systems, Minneapolis, MN) was reconstituted in 4 mM HCl containing 1 mg/ml BSA (Sigma Chemical Co.) and stored at −20°C.

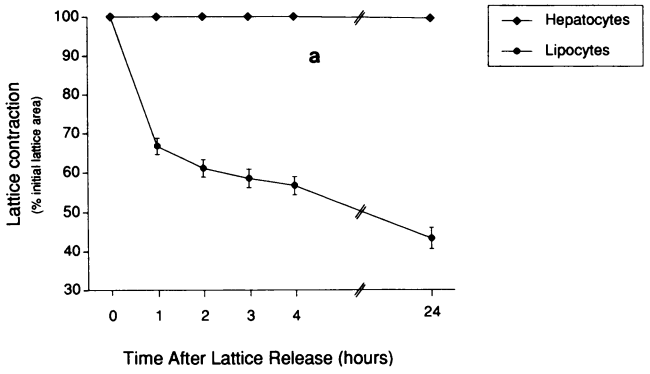
Microscopy. Phase contrast and immunofluorescence photomicrographs were taken with a Diaphot Inverted Microscope (Nikon Inc. Instrument Group, Garden City, NY) using Ilford Plus film (Ilford

Ltd., Cheshire, UK). For transmission electron micrographs, collagen lattices were washed twice in PBS and fixed for 30 min with 2% glutaraldehyde in HBSS (Gibco Laboratories) and washed twice in HBSS. Collagen lattices were post-fixed in reduced 2% osmium tetroxide for 1 h and subsequently dehydrated through graded ethanol solutions. Small strips of sample were infiltrated and embedded in Eponate 12 resin, 60-nm sections were poststained with 2% uranyl acetate (aqueous) and lead citrate, and examined with a 10 C electron microscope (Carl Zeiss, Inc., Thornwood, NY). Photomicrographs were taken with Kodak EM 4489 film (Eastman Kodak Co., Rochester, NY). For scanning electron microscopy, collagen lattices were washed twice in PBS and fixed for 1 h with 2% glutaraldehyde in HBSS containing 20 mM Hepes, pH 7.4, and washed twice in HBSS. Collagen lattices were post-fixed in reduced 1% osmium tetroxide for 1 h, washed twice with water, and dehydrated through graded ethanol solutions. Samples were critical point-dried, sputter-coated with platinum-gold, and viewed on an ISI DS-130 scanning electron microscope (Topcon Technologies, Inc., Pleasanton, CA) at 15 kV. Photomicrographs were taken with Kodak EM 4489 film (Eastman Kodak Co.). Unless otherwise stated, all electron microscopy reagents were obtained from Pellco Inc., Redding, CA.

Statistics. Statistics were performed using Student's paired t test. Each experiment utilized cells from a different animal. The average of duplicate cultures from each experiment generated a single data point. In the calculation of mean values and statistical variation, n refers to the number of separate experiments (i.e., individual cell preparations) unless stated otherwise. Error bars depict the SEM; absence of error bars indicates that the SEM was < 1%.

Results

Contraction of collagen lattices by isolated lipocytes and hepatocytes. In initial studies, lipocytes from normal rats were isolated and cultured on type I collagen lattices in medium containing 20% (1:1 horse and calf) serum for 5 days. Lipocytes induced rapid and sustained contraction after lattice detachment (Fig. 1 *a, closed circles*). Lattice area was reduced to 43.7%±2.3% of initial size 24 h after detachment (Fig. 1 *a* and *b*). An additional small increment in contraction was evident 48 h after lattice detachment; lattice area remained unchanged thereafter (not shown). There was no contraction of cell-free lattices (not shown) or of those containing hepatocytes (Fig. 1).



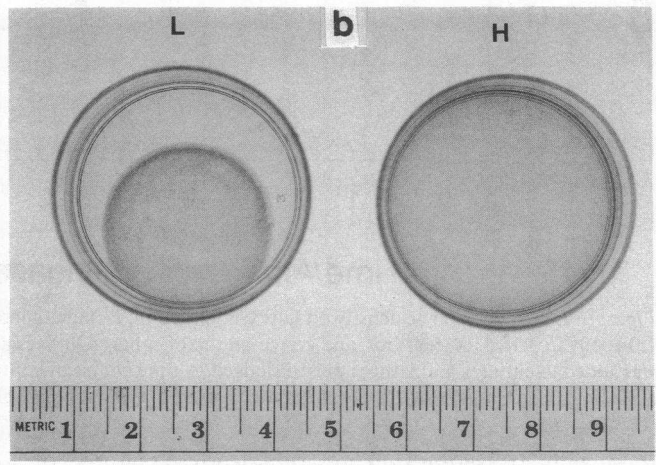


Figure 1. Lipocyte and hepatocyte contraction of collagen lattices. (*a*) Lipocytes were isolated and cultured on collagen lattices in medium containing serum. The lattices were detached from culture dishes on day 5. Hepatocytes (10^6 cells/ml, *closed diamonds*) were cultured on collagen lattices under similar conditions. Contraction was measured as the reduction in the initial lattice area over time and, for hepatocytes vs. lipocytes (*closed circles*) in serum $P < 0.001$ for all time points ($n = 5$). (*b*) Lipocytes (*L*) and hepatocytes (*H*) cultured on 35-mm collagen lattices were dislodged as described in Methods. Lattices are shown 4 h after release, at ×1.

Lipocyte contraction of collagen lattices was proportional to cell density at plating concentrations of $\leq 2.5 \times 10^5$ cells/ml, and was maximal at 2.5×10^5 to 5×10^5 cells/ml (Fig. 2). No additional increment in contraction was evident at higher densities.

Contractility of lipocytes cultured within collagen lattices was also examined. After lattice detachment, contraction occurred at a slower rate (i.e., over a more prolonged time period) than for cells cultured on top of collagen lattices; maximal contraction occurred at 72 h (not shown). In all other respects, the behavior of lipocytes within collagen lattices was identical to that for cells on top of lattices.

Morphology of contractile lipocytes. Lipocytes plated either on top of or within collagen lattices acquired a stellate appearance after 48–72 h in primary culture, which was maintained for up to 2 wk (Fig. 3 *a*). Additional features of activation, including nuclear enlargement and loss of retinoid droplets, as previously observed in lipocytes cultured on uncoated plastic or glass (29), were evident. Activation was further documented by immunolocalization of smooth muscle α actin in lipocytes, beginning at 3 d in culture (10), but is not shown. Lipocytes induced alignment of collagen fibrils consistent with tractional structuring, as previously reported in fibroblasts (35). Transmission electron microscopy demonstrated abundant thin filaments in culture-activated lipocytes. Phase contrast microscopy during collagen lattice contraction revealed a prominent reduction in lipocyte cell volume (Fig. 3 *b*) and development of conspicuous cell–cell cytoplasmic extensions (Fig. 3 *b*).

Scanning electron microscopy of lipocytes that were isolated from normal rats in the presence of serum and maintained in culture for 5 d demonstrated a characteristic spread appearance (Fig. 4 *a, inset*). Intimate cell–collagen associations were readily observed (Fig. 4, *inset*). Occasional cytoplasmic processes of adjacent cells were closely associated, but are not shown here. During contraction, cytoplasmic volume was markedly reduced (Fig. 4 *b*).

Effects of serum and soluble mediators on lipocyte contraction. In 5-d-old cultures maintained in serum-containing medium, replacement with serum-free medium for 4 h (three changes of medium), resulted in markedly diminished contraction after lattice detachment (Table I). Re-introduction of me-

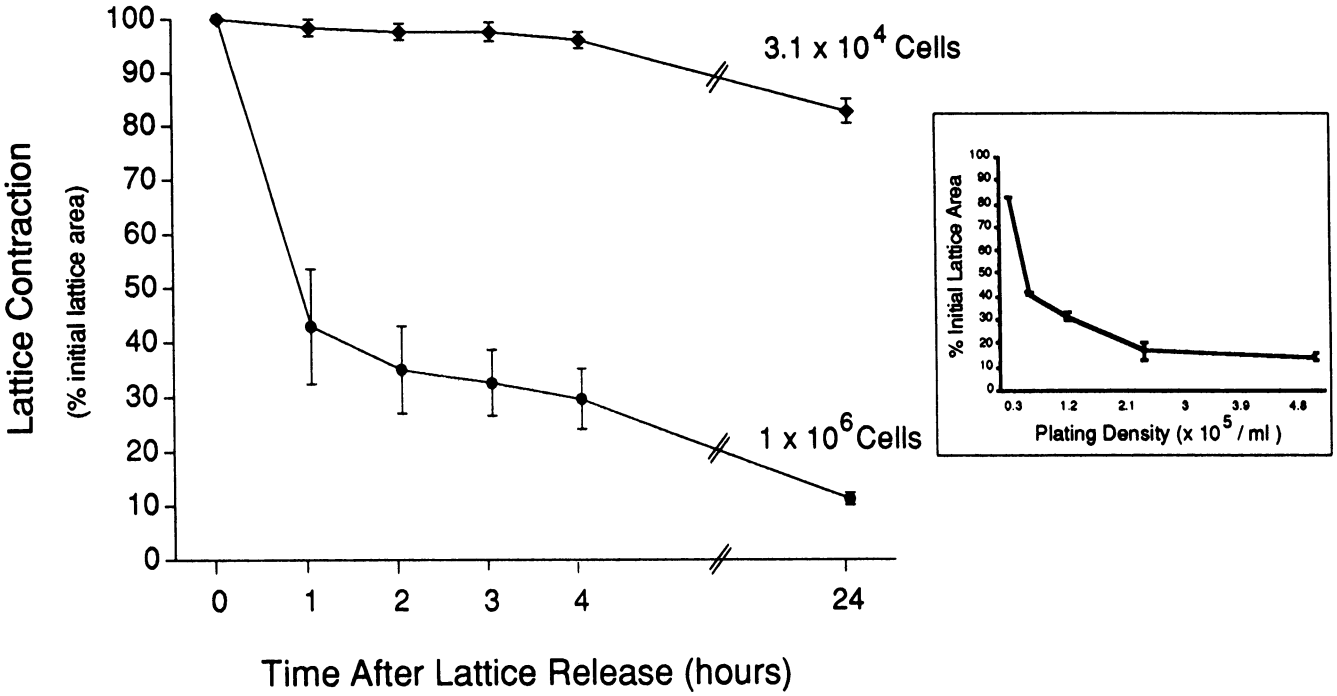


Figure 2. Effects of lipocyte density on lattice contraction. Isolated lipocytes were diluted in plating medium plus 20% serum to concentrations of 1.0×10^6 to 3.1×10^4 cells/ml, and grown on top of collagen lattices as described in Methods. After 5 d in culture, endothelin-1 (2×10^{-8} M) was added to cultures and lattices were dislodged. A time course of contraction for the maximal cell density is shown, relative to the minimally effective cell density. The relationship of cell density to contraction is also shown (*inset*).

dium with 20% serum back to serum-deprived cultures completely restored contractile activity (Table I). Furthermore, contraction was linearly related to serum concentrations between 1 and 20% ($r = 0.964$, not shown). Plasma (1:1 horse/calf) had the same effect as serum (not shown).

The contractile response of lipocytes to several putative vasoactive substances was tested (Table I). These studies were performed in serum-free conditions to avoid background contraction caused by serum. Endothelins 1, 2, and 3 (all 2×10^{-8} M) were equally active. Neither the vasoactive peptides angiotensin II, thrombin, or 5-hydroxytryptamine, nor cytokines PDGF and TGFβ induced significant contraction (Table I).

We excluded the possibility that endothelin in serum was responsible for lipocyte-mediated lattice contraction, because the endothelin-1 concentration in serum was 1.9×10^{-12} M$\pm 0.11 \times 10^{-12}$ M, as measured by RIA. This concentration was only 1/100 the minimal active concentration in our system (not shown). Additionally, size fractionation using Centricon 10 and 30 centrifugal microconcentrators (Amicon, Inc., Beverly, MA) demonstrated that the molecular size of the substance(s) in serum was above 10–12 kD, a size far greater than any of the endothelins.

Additive effects of serum and endothelin-1. We compared contraction induced by serum or endothelin-1 alone to their activity in combination. After standard culture for 5 d, lattices were incubated with serum-free medium for 4 h (three washes) and serum-free medium alone, medium containing serum (20%), serum-free medium plus endothelin-1 (2.0×10^{-8} M), or medium containing serum and endothelin-1 were added. When both agents were tested simultaneously, their effects were additive, inducing contraction to 22.6%±0.9% of initial lattice area at 24 h ($P < 0.05$ and 0.005, vs. endothelin alone and serum alone, respectively; $n = 4$). The additive effect of serum and endothelin-1, observed 24 h after lattice release, was also present at 2, 4, and 48 h (not shown).

Lipocyte contractility is dependent on an intact cytoskeleton. The cytoskeletal disrupting agent cytochalasin B, inhibited serum- and endothelin-1–induced lattice contraction in a dose-dependent manner, as presented in Table II ($r = 0.994$ for concentrations from 0 to 5 μg/ml). Cytochalasin B exposure led to disappearance of the highly conspicuous cell–cell interactions that had become apparent during contraction, as noted in Fig. 3 *b*.

Lipocyte contractility requires cellular activation. We initially observed that several days of culture were required before lipocytes from normal rats were contractile. To determine whether contractility was dependent on cellular activation, we examined the relationship of smooth muscle α actin, an established marker of lipocyte activation (10), to contraction (Fig. 5). In lipocytes from normal liver, expression of smooth muscle α actin increased over the first 5 d in culture (Fig. 5, *upper panel*). Furthermore, smooth muscle α actin expression by these lipocytes correlated with contractility during progressive primary culture (Fig. 5, *lower panel*). Contraction, 4 h after lattice detachment at 1, 3, 5, 7, 10, or 14 d in culture was: day 1, 100±0.0%; day 3, 83.9±6.2; day 5, 58.8±7.3 ($P < 0.001$ day 1 vs. day 5); day 7, 47.0±3.3; day 10, 46.5±5.1; day 14, 55.0±2.8; data represent the percent of initial lattice area±SEM ($n = 4$). There was no statistical difference in contraction between days 5, 7, 10, or 14. Contraction induced by endothelin-1 paralleled that shown for serum at the various time points (not shown).

To further establish that lipocyte contractility was activation dependent, we tested the effects on contraction of IFN-γ, an agent we have previously shown to inhibit lipocyte activation (36). IFN-γ markedly attenuated the contractile effects of

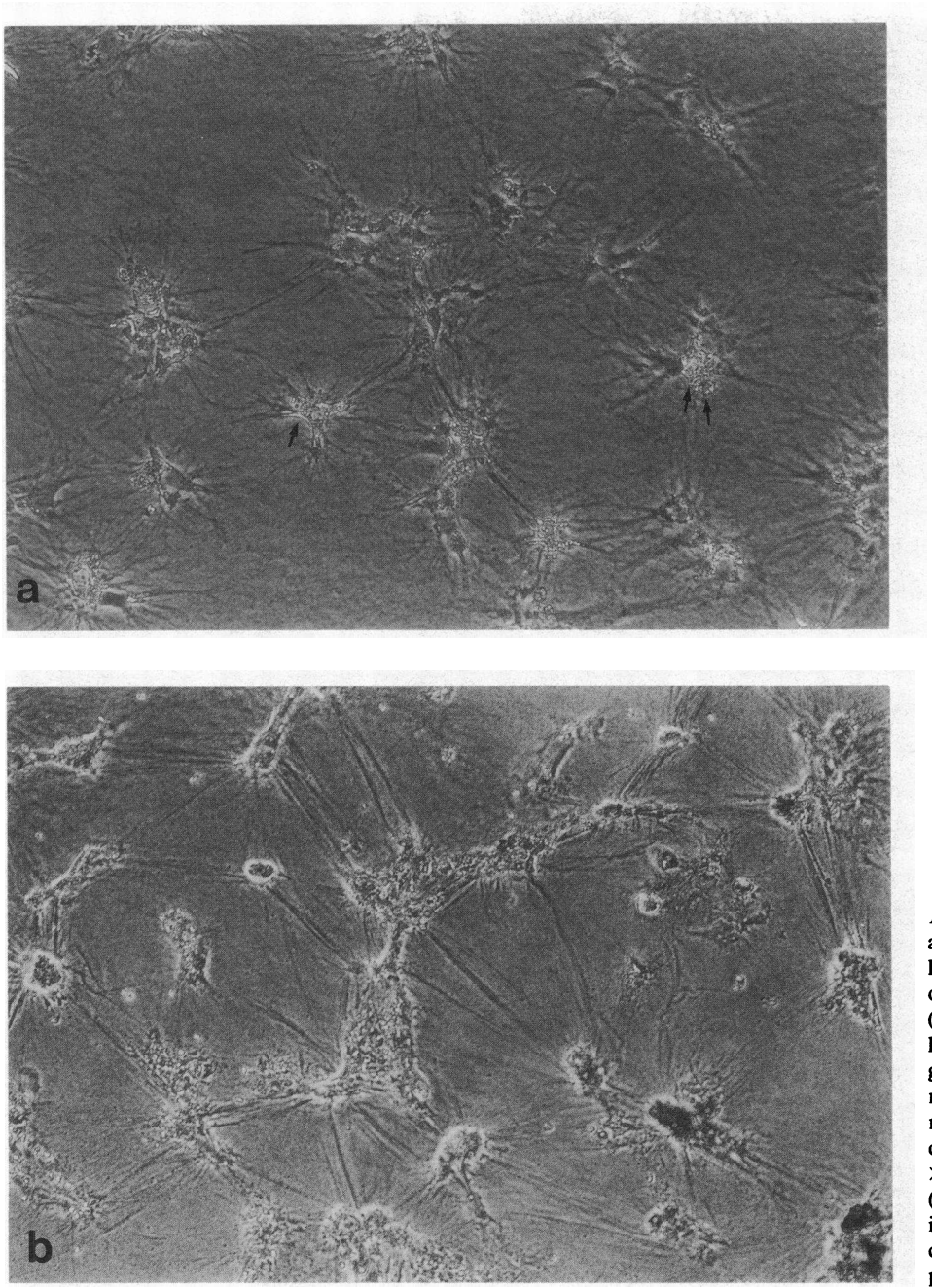


Figure 3. Phase contrast morphology of activated lipocytes cultured on collagen lattices. Lipocytes were cultured for 5 d on top of collagen lattices in serum (20%) containing medium. (*a*) Before lattice detachment, cells displayed elongated cytoplasmic processes, enlarged nuclei, and prominent perinuclear retinoid droplets (*arrows*) typical of lipocytes. The initial plating density was $2.5 \times 10^5/mm^3$. (*b*) 4 h after lattice release (during active contraction), reduction in cytoplasmic area and association of cell–cell cytoplasmic extensions is prominent. Both ×320.

serum and endothelin-1 (Fig. 6) with no effect on cell morphology, viability, or number.

We examined the behavior of lipocytes isolated from rats treated with CCl_4 to establish the relevance of our culture model to liver injury in vivo. After 8 wk of weekly dosing with CCl_4, livers used for cell isolations appeared nodular and were palpably firm. Cells from these animals displayed features of markedly accelerated activation upon culturing. Lipocytes isolated from fibrotic livers spread immediately (not shown), and developed an activated appearance within 12–24 h, whereas cells from normal rats required 4–5 d in culture to acquire a similar appearance. Dual immunocytochemical studies (24 h after plating) were performed with lipocytes from normal and CCl_4-treated animals for desmin and smooth muscle α actin (Fig. 7). Whereas desmin-positive lipocytes from normal rats did not express smooth muscle α actin, lipocytes from fibrotic animals abundantly expressed this protein (Fig. 7). Immunoblotting, normalized for total protein, confirmed a high degree of activation in this population of lipocytes as reflected by the substantial expression of smooth muscle α actin in lipocytes from CCl_4-treated animals (Fig. 8).

We also examined contractility of freshly isolated lipocytes from normal and CCl_4-treated rat livers. In contrast to lipocytes from normal rats, activated cells from fibrotic animals were contractile immediately after adherence (Fig. 9). Contractility of cells from fibrotic animals persisted at a uniform level for at least 5 d after plating (not shown).

Discussion

The possibility that lipocytes are contractile has been raised by several lines of evidence: (*a*) their expression of the interme-

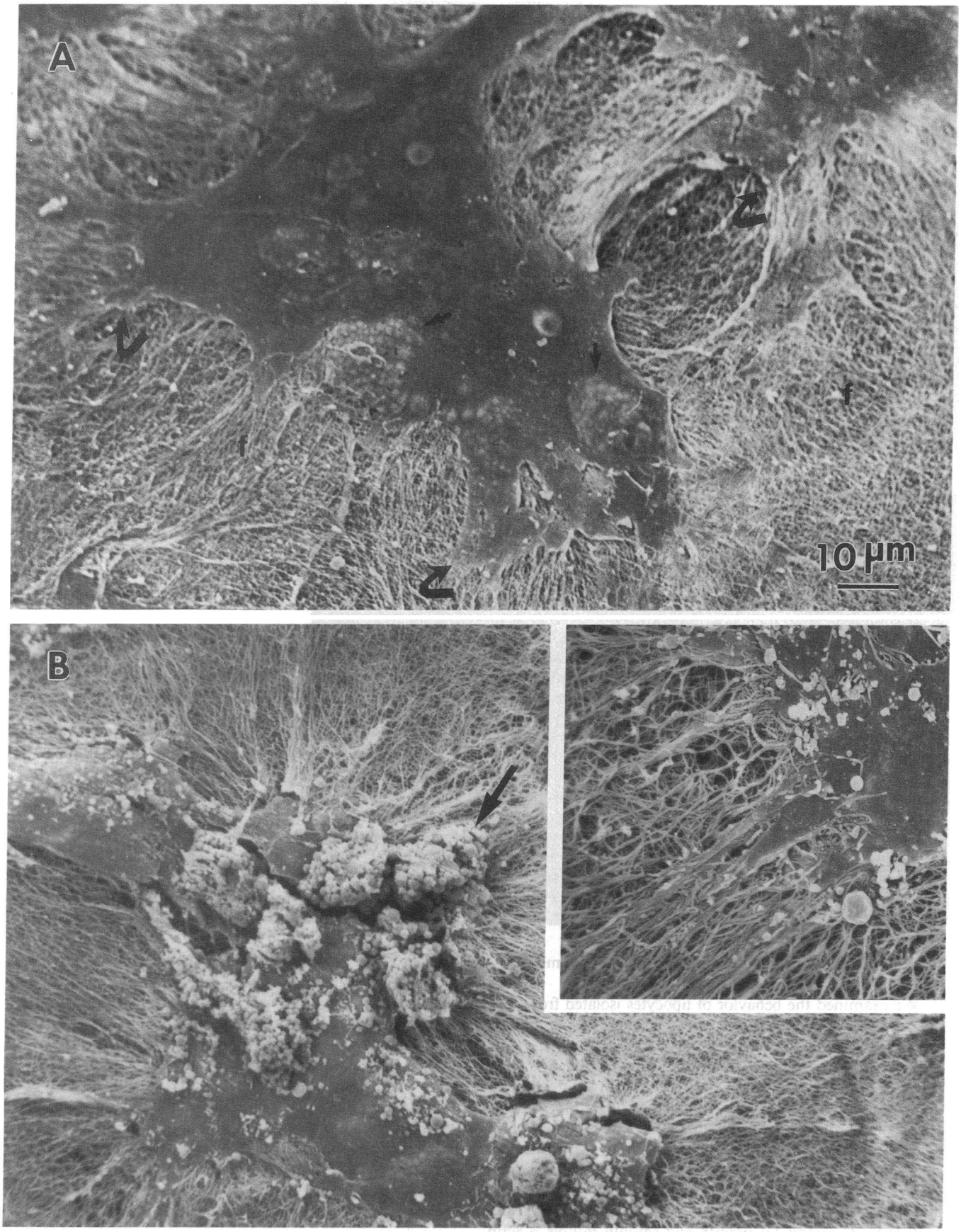


Figure 4. Scanning electron microscopy of contractile lipocytes on collagen lattices. After 5 d in culture before lattice release (*A*), lipocyte cytoplasmic processes (*curved arrows*) extend over collagen fibrils (*f*). Retinoid droplets (*small arrows*) are evident under the cell membrane. 4 h after release of the lattice (*B*), a decrease in cytoplasmic area and retraction of collagen fibrils are conspicuous during contraction. Extrusion of intracellular rounded bodies (*curved arrows*) is conspicuous. Before and during contraction, intricate association of lipocyte cytoplasmic cell processes and collagen fibrils is also evident (*inset*). *A* and *B* ×1,200; *inset* ×3,000.

Table I. Effect of Serum, Vasoactive Agents, and Cytokines on Lipocyte Contraction

Mediator	Contract (±SEM) (% of initial lattice area)	*n*	*P* value Mediator vs. control
Medium + 20% serum	48.9±3.0	17	<0.0005
Control	90.6±1.6		
Angiotensin II (8.6 μM)	89.4±2.9	3	NS
Control	90.4±3.8		
Thrombin (10 U/ml)	94.8±3.9	3	NS
Control	91.6±4.0		
Serotonin (10 mM)	94.6±1.6	3	NS
Control	92.5±3.3		
Endothelin-1 (20 nM)	38.9±3.3	7	<0.01
Control	93.3±2.9		
Endothelin-2 (20 nM)	40.5±4.2	3	<0.01
Control	87.9±5.5		
Endothelin-3 (20 nM)	40.5±4.2	3	<0.01
Control	87.9±4.2		
PDGF (40 ng/ml)	95.7±2.9	3	NS
Control	96.9±1.8		
TGF-β (2 ng/ml)	96.8±2.6	3	NS
Control	95.9±2.8		

After culture for 5 d in serum-containing medium (20% serum), lipocytes were incubated in serum-free medium for 4 h (3 washes). Mediators were added in serum-free medium and lattices were released (detached). Contraction was measured as a reduction in lattice area over time; the recorded values are those after 24 h. Controls consist of parallel cultures from the same cell isolation maintained in serum free medium. For all preparations, serum induced contraction (lattice area reduced to < 55% original size, not shown). Thrombin activity was confirmed by demonstrating conversion of fibrinogen (1 mg/ml) to fibrin (60). The activity of angiotensin II was confirmed by its ability to increase portal pressure in the perfused rat liver (61; Rockey, D. C., unpublished observation). TGF-β and PDGF activity was verified by their ability to induce rat kidney fibroblasts to contract collagen lattices as described in Methods.

diate filament, desmin assigns them to a muscle-cell lineage (20–21); (*b*) they are intimately associated with the endothelium in a perisinusoidal distribution (37) suggesting that lipocytes are the liver analogue of pericytes (38). Pericytes are purportedly related to vascular smooth muscle cells and thought to

Table II. Effect of Cytochalasin B on Lipocyte Contraction

Cytochalasin B	Contraction (±SEM) (% initial lattice area)	*P* value (*n* = 4) (vs. "0")
μg/ml		
0	23.9±2.3	—
1	54.1±0.5	<0.005
2.5	83.4±4.1	<0.005
5	99.0±1.0	<0.001
10	100.0±0	<0.001

Lipocytes were cultured in serum containing medium for 5 d then incubated with cytochalasin B for 2 h. Endothelin-1 (2×10^{-8} M) was added and lattices were released (detached). Contraction was measured as reduction in lattice area over time and is shown 24 h after release.

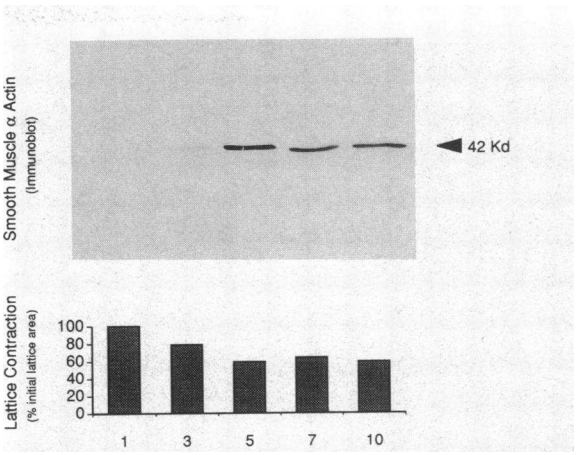


Figure 5. Culture-induced activation is associated with development of contractility. Lipocytes on 35-mm collagen lattices were cultured in the presence of medium containing serum for 1, 3, 5, 7, or 10 d, then released. Contraction was monitored for 4 h (*lower panel*). The same lattices were liquefied with collagenase and the cells were collected and probed with anti-smooth muscle α actin antibody (*upper panel*) as described in Methods. The data shown in this figure are from a representative experiment.

play a role in regulation of capillary blood flow (22–24); (*c*) lipocytes express smooth muscle specific α actin under some conditions (9, 10).

Investigation of cellular contraction using collagen lattices (gels) was introduced by Bell and colleagues and has been validated as a model for study of cellular contraction in a variety of mesenchymal cell types (30, 39–44). For example, fibroblasts seeded on top of type I collagen gels bind and organize underlying collagen fibrils, both in close proximity and at distance from cells (39). After such reorganization, lattices are converted into a dense mass with the morphological appearance of dermal scar (39).

A major point established by this work is that lipocytes become contractile only after activation, as indicated by mor-

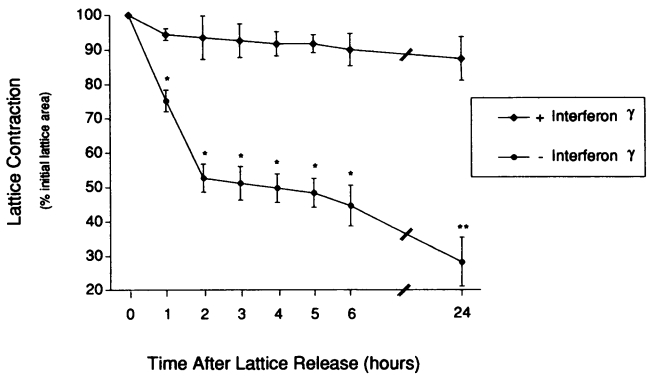


Figure 6. Effect of IFN-γ on lipocyte contraction. Lipocytes were isolated and cultured on 35-mm lattices (*closed circles* indicate the absence of IFN-α) in serum containing medium. IFN-γ (1,000 U/ml) was added (*closed diamonds*) to lipocyte cultures 24 h later and renewed with medium changes. After 5 d in culture, lattices were detached and contraction was monitored in the presence of serum and endothelin-1 (2×10^{-8} M). $^{*}P < 0.05$; $^{**}P < 0.001$; ($n = 3$).

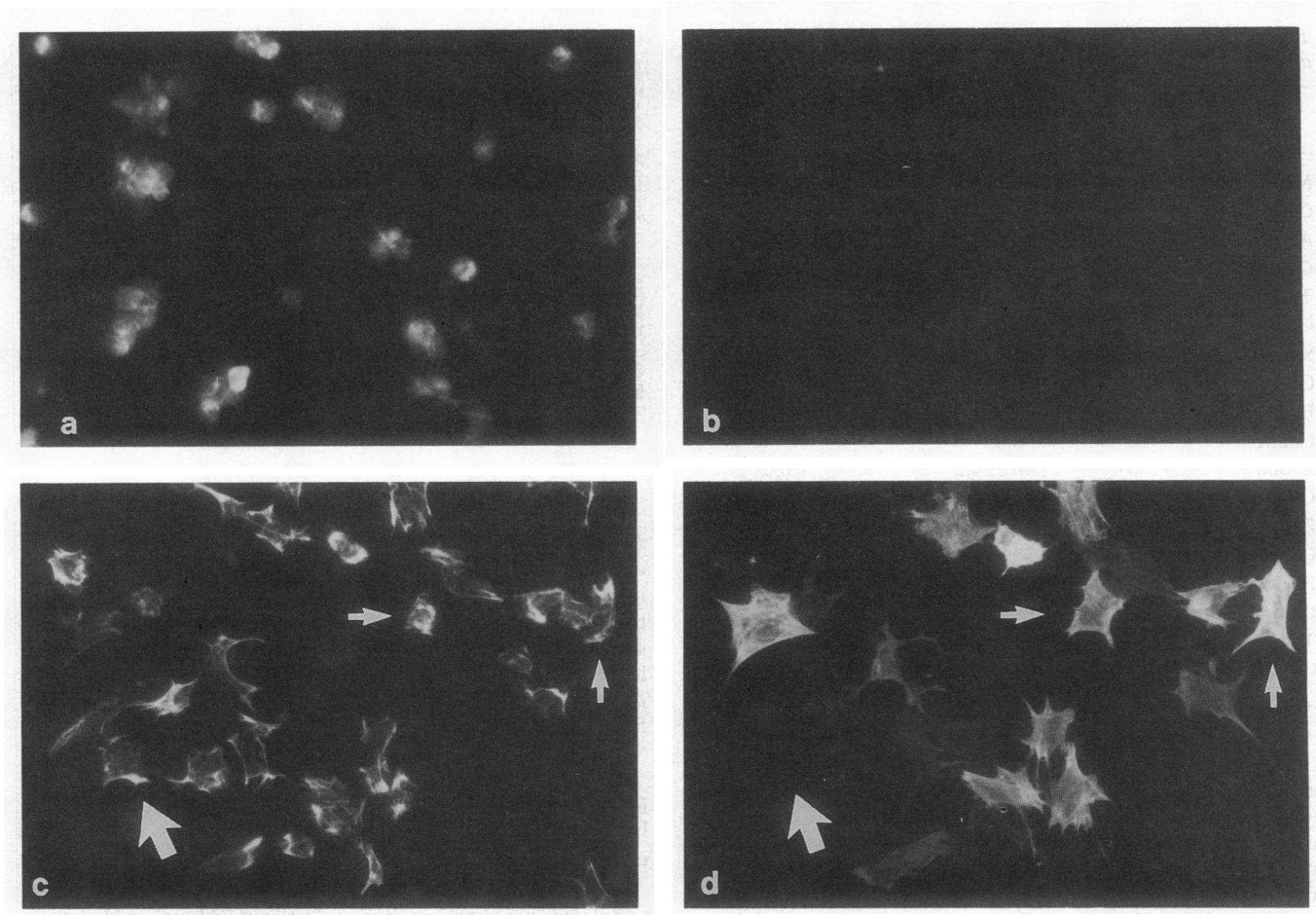


Figure 7. Smooth muscle α expression in lipocytes from normal and fibrotic liver. Lipocytes were isolated from normal and fibrotic liver as in Methods. After plating, cells were fixed and probed with anti-desmin and anti–smooth muscle α actin antibodies. Dual immunofluorescence reveals that, 24 h after plating, lipocytes isolated from normal rat liver were compact and expressed desmin (*a*), but not smooth muscle α actin (*b*). In contrast, 8 h after isolation, lipocytes from fibrotic liver (after 8 wk with CCl_4) were spread, expressed desmin (*c*), and smooth muscle actin (*d*). Most cells expressed both desmin and smooth muscle α actin (*small arrows*); some cells expressed desmin but not smooth muscle α actin (*large arrows*). Identical results were observed in three separate experiments. Control specimens incubated with irrelevant mAb and non-immune rabbit IgG displayed no significant staining (not shown). a–d, ×200.

phologic changes and appearance of smooth muscle α actin (9, 10). The inhibitory effect of IFN-γ (36) in cultured lipocytes further reinforces the activation dependence of lipocyte contraction, since this agent inhibits features of activation in culture. We have demonstrated that lipocytes activated in vivo are contractile immediately upon plating. This finding establishes that contractility in our model is relevant to liver injury in vivo and is not simply a culture-related artifact. Additionally, our studies of lipocyte contraction on type I collagen matrices are particularly well suited as a model for liver injury because this collagen type predominates in the subendothelial space of Disse during fibrogenesis (27). Lipocytes, which reside in this

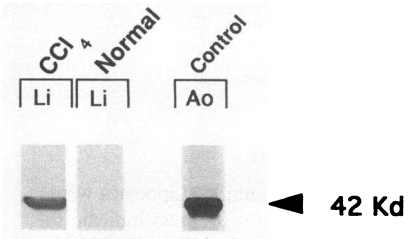


Figure 8. Immunoblot of smooth muscle α actin in lipocytes from normal and fibrotic liver. Protein extracts (all 20 μg of total protein) from normal rat aorta, and from lipocytes isolated from normal and CCl_4 treated rats (8 wk) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti–smooth muscle α actin antibody as in Methods. Identical results were observed in three separate experiments. A representative immunoblot is shown.

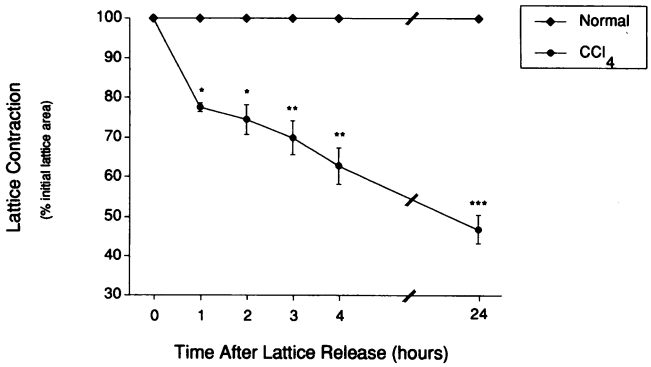


Figure 9. Contractility of lipocytes from fibrotic liver. Lipocytes were isolated from normal (*closed diamonds*) and CCl_4-treated rats (*closed circles*) and cultured on collagen lattices in medium containing serum. After adherence (i.e., 24 h after plating), lattices were detached and their area measured. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; ($n = 3$).

space, are thus optimally situated to induce liver (wound) contraction via interaction with this milieu rich in type I collagen.

Although the cellular components that confer contractility are not yet identified, the concurrent induction of smooth muscle α actin suggests a direct role of this protein in the contractile response. By immunoblot analysis, smooth muscle α actin expression parallels changes in contractility at all stages examined in culture. However, direct evidence that smooth muscle α actin is a contractile element awaits development of a method to selectively inhibit function of this protein without affecting other features of activation.

Our data raises fundamental questions about the lineage and function of lipocytes and related terminal perivascular cells. Normal lipocytes and those in early culture (i.e., quiescent, "not activated") are not contractile. Yet lipocytes are felt to represent liver specific pericytes (22–24, 45), which are supposed to be vasoregulatory (24, 25). In fact, a contractile role for pericytes has not been established in all tissues (22, 24, 46, 47). Although capillary pericytes contain smooth muscle specific contractile proteins (48) and isolated pericytes contract in vitro (43, 49), evidence of their contractility in vivo is limited (24). For example, Tilton (47) and co-workers demonstrated in vivo wrinkling of skeletal muscle, but not cardiac pericytes, in response to vasoactive agents; moreover, the authors were unable to exclude a contribution by endothelial cells. Our finding that normal (quiescent) lipocytes do not contract supports the view that not all pericytes are contractile. Alternatively, lipocytes may not be true pericytes. Since lipocytes acquire morphological features of myofibroblasts, display smooth muscle markers, and exhibit contractility only during activation, it seems more likely that they represent "pericyte-like" mesenchymal cells that retain the potential to transform into contractile myofibroblasts during injury.

In activated lipocytes, contractility was elicited only by endothelins, but not by other contractile agonists. Thrombin, angiotensin II, serotonin, PDGF, and TGF-β were not active in the absence of serum. The lack of activity of thrombin and angiotensin II contrasts with recent data of Pinzani and colleagues (25), who demonstrated an increase in intracellular free Ca^{2+} concentrations in passaged human lipocytes in response to these agents. There are several potential reasons for this discrepancy. First, rat lipocytes may differ in some respects from their human counterparts. In particular, unlike rat cells, which express desmin uniformly, desmin content in human lipocytes is variable and may be minimal to absent (51–52). Additionally, while rat lipocytes express smooth muscle α actin only with activation, human lipocytes abundantly express this protein, even in normal liver (52). Second, Pinzani and coworkers utilized passaged cells, whose characteristics are likely to differ substantially from primary cell cultures (53). Third, although ATP-dependent uptake of Ca^{2+} induced by inositol triphosphate (54) implies contractile behavior, changes in Ca^{2+} may not always indicate active contraction. For example, Ca^{2+} influx in response to inositol 1,4-biphosphate and inositol 1,4,5 triphosphate has been demonstrated in noncontractile cells such as pancreatic acinar and myeloid cells (55, 56). Finally, cell rounding as described by Pinzani and co-workers may not necessarily reflect contraction, as rounding can also occur in response to toxic mediators or disruption of cell–matrix interactions (57).

Serum contains specific but as yet unidentified elements, which stimulate lipocyte (and fibroblast) contraction (58). Because plasma and serum had similar effects, it seems unlikely that the contractile agonist is a platelet factor (or factors), and indeed PDGF was inactive. Our preliminary studies suggest that the material has a molecular mass of greater than 10–12 kD and is not an endothelin. In addition, the additive effects of serum and endothelin-1 suggest that these two substances mediate contraction by different pathways. It remains possible, however, that serum present at the time of plating and in early culture may enhance stress fiber and focal adhesions as recently reported in fibroblasts (59), and thus augment endothelin-mediated contraction.

In summary, we have demonstrated that rat hepatic lipocytes exhibit a contractile phenotype upon activation either in culture or in vivo. This finding has important implications for the pathophysiology of portal hypertension. Because of their perisinusoidal location, lipocytes are ideally situated to regulate sinusoidal blood flow. However, our data indicate that the contractile phenotype is confined to activated cells, implying that lipocytes may modulate hepatic blood flow only during liver injury. In this setting, activated lipocytes may induce contraction of fibrotic bands, leading to physical distortion of lobular architecture and secondary disruption of normal hepatic blood flow patterns. Alternatively, hepatic blood flow could be altered by perisinusoidal constriction. The development of activation-inhibiting agents such as IFN-γ and/or specific contractile antagonists may represent potential points of therapeutic intervention in portal hypertension associated with chronic liver injury.

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