Rat Hepatocytes in Serum-free Primary Culture Elaborate an Extensive Extracellular Matrix Containing Fibrin and Fibronectin

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Abstract. Adult rat hepatocytes cultured on type IV collagen, fibronectin, or laminin and maintained in serum-free medium were examined by indirect immunofluorescence using polyclonal antibodies against extracellular matrix proteins. An extensive fibrillar matrix containing fibronectin and fibrin was detected in all hepatocyte cultures irrespective of the exogenous matrix substratum used to support cell adhesion. Fibrils radiated from the cell periphery and covered the entire culture substratum. In addition, thicker fibers or bundles of fibers were localized on top of hepatocytes. This matrix did not contain laminin or the major types of collagen found in the liver biomatrix (types I, III, and IV). Isolation of the fibrillar matrix and analysis on polyacrylamide gels under reducing conditions demonstrated a major 58-kD polypeptide, derived from β-fibrinogen as indicated by immunoblotting and two-dimensional peptide mapping. Plasmin rapidly dissolved the matrix. Deposition of the fibrin matrix in hepatocyte cultures was arrested by hirudin, by specific heparin oligosaccharides that potentiate thrombin inhibition by antithrombin III, and by dermatan sulfate, an activator of heparin cofactor II-mediated inhibition of thrombin. The results indicate that hepatocytes in culture synthesize and activate coagulation zymogens. In the absence of inhibitory and fibrinolytic mechanisms, a fibrin clot is formed by the action of thrombin on fibrinogen. Fibronectin attaches to this fibrin clot but fails to elaborate a fibrillar matrix on its own in the presence of coagulation inhibitors.

THE relation between the hepatic extracellular matrix (17, 28, 38) and normal functions of the liver parenchyma is evident from pathological conditions that manifest abnormal and disorderly matrix accumulation (17, 39, 40). Isolated hepatocytes can be maintained in vitro on a variety of biomatrix protein substrata, offering an excellent system to study cell–matrix interactions. We have recently used fibronectin, type IV collagen, and laminin substrata to support serum-free growth of rat hepatocytes and have reported on substratum-specific morphological (6, 30) and functional (42) effects.

In this report, immunofluorescence microscopy and a variety of polyclonal antibodies were used to study the architecture of the extracellular matrix produced by primary hepatocyte cultures grown on type IV collagen, laminin, or fibronectin substrata. Our major finding is that rat liver hepatocytes synthesize an extensive fibrillar matrix in vitro consisting of polymerized fibrin and fibronectin. The matrix did not contain any collagenous components and was equally prominent in all hepatocyte cultures, irrespective of the substratum used to support cell adhesion. Formation of the matrix was inhibited by anti-clotting agents such as heparin, hirudin, and dermatan sulfate.

Materials and Methods

Cells

Hepatocytes were isolated from young adult female Sprague-Dawley rats by liver perfusion (5) and cultured in a basal arginine-free DME (45) supplemented with ornithine (0.5 mM), insulin (0.1 μM), dexamethasone (0.025 μM), penicillin (60 μg/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml). Cells were plated onto plastic petri dishes or glass coverslips coated with type IV collagen (40 μg/ml), fibronectin (30 μg/ml), or laminin (40 μg/ml) for 1 h at 37°C. When glass substrata were used, the matrix proteins were either passively absorbed or covalently linked to the glass (2) with no apparent difference regarding the effect on the cell culture. Serum-free medium was used throughout when matrix protein substrata were used. Hepatocytes were also plated onto plain glass or plastic substrata in medium supplemented with 20% FCS. 2 h after inoculation the medium was discarded and the culture was thereafter maintained in serum-free medium.

Proteins and Glycosaminoglycans

Fibronectin was isolated from rat plasma on a gelatin-Sepharose column (14). Type IV collagen and laminin were isolated from the Engelbreth-Holm-Swarm sarcoma implanted in lathyritic mice as described (20). Rat fibrinogen, antithrombin III, plasmin, heparin, dermatan sulfate, hirudin, and bacterial collagenase (Sigma type IV) were purchased from the Sigma Chemical Co. (St. Louis, MO). Heparin fragments with high or low affinity for antithrombin III were prepared by partial deamination of heparin with nitrous acid, fractionation on antithrombin III-Sepharose, and gel filtration (24, 48).

Antibodies

Rat plasma fibronectin antibodies were raised in rabbits in this laboratory. Type IV collagen and laminin antisera were raised in rabbits by Dr. D. M. Bissell (San Francisco General Hospital, San Francisco, CA). Sheep anti-rabbit fibrinogen was a gift from Dr. P. Gaffney (National Institute of Biological Standards and Control, London, England) and goat anti-rat fibrinogen was purchased from Sigma Chemical Co. Antibodies against collagen type I and III were kindly provided by Drs. M. Pope and A. Temple (Clinical Research Centre, Northwick Park, London). Fluorophore-conjugated antibodies used were the following: goat anti-rabbit IgG/rhodamine (Cappel Laboratories, Inc., Cochranville, PA), goat anti-rabbit/fluorescein isothiocyanate (Miles-Yeda, Rehovot, Israel), rabbit anti-sheep/fluorescein (Dr. M. Parkhouse, National Institute for Medical Research, London), donkey anti-sheep IgG/fluorescein, horseradish peroxidase–conjugated anti-rabbit IgG and anti-goat IgG (Sigma Chemical Co.).

Indirect Immunofluorescence

Hepatocyte cultures on 13-mm-diam coverslips were fixed in freshly made paraformaldehyde for 20 min at room temperature. After extensive washing they were incubated with antiserum for 1 h at room temperature, thoroughly rinsed, and incubated with the appropriate fluorophore-conjugated second antiserum for 1 h at room temperature. Both antibodies were diluted 1:40 in PBS. The coverslips were exhaustively rinsed in PBS and mounted on microscope slides in glycerol/PBS (9:1) containing phenylene diamine (1 mg/ml). In double-labeling experiments, sheep anti-rabbit fibrinogen was used together with rabbit anti-rat fibronectin (1:40 and 1:100 final dilutions, respectively), followed by rhodamine-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG and fluorescein-conjugated donkey anti-sheep IgG (1:40 final dilutions). The fluorescein-conjugated donkey anti-sheep IgG (batch No. 44F-8813; Sigma Chemical Co.) showed some reactivity with rabbit immunoglobulins. Therefore, in order to avoid nonspecific interactions with the rabbit anti-rat fibronectin antibody, the fluorescein-conjugated antibody was pretreated with nonimmune rabbit serum for 15 min at room temperature and then incubated with protein A-Sepharose 4B for 30 min at room temperature. The supernatant was removed and used in the double-labeling experiments. This treatment did not affect the reactivity of the antibody with sheep IgG indicating that in comparison with rabbit IgG, donkey IgG has a reduced affinity for protein A. Matrix immunostaining with anti-fibrinogen was achieved in both double-label (using sheep anti-rabbit fibrinogen) and in single-label (using goat anti-rat fibrinogen) experiments. Both fibrinogen antibodies reacted with reference standard fibrinogen in immunoblots.

Controls in immunofluorescence experiments included omission of the first or second antibodies, use of preimmune sera, and absorption of the fibronectin or fibrinogen antibody with the respective antigen. All controls were negative. The slides were examined in a Zeiss ICM 405 inverted microscope equipped for epifluorescence.

Matrix Isolation

The hepatocyte matrix was isolated from hepatocytes grown for 40 h on plastic petri dishes. The culture was rinsed and, using a drawn-out Pasteur pipette, long bundles of fibrils attached to dorsal cell surfaces were removed and solubilized in Laemmli's electrophoresis sample buffer (23). The whole procedure was monitored with an inverted microscope. Some cells were inevitably dislodged and removed together with the matrix but their mass was small in relation to that of the matrix.

Metabolic Labeling

Labeling of hepatocyte cultures with [^{35}S]methionine was performed as described earlier (42). Briefly, cells were labeled for 20 h with 25 μCi/ml of [^{35}S]methionine in basal medium containing 1% of the usual amount of methionine.

PAGE

Electrophoresis of protein samples was performed in 5–12% gels using the buffer system of Laemmli (23). Running buffers contained SDS and samples were reduced with β-mercaptoethanol.

Immunoblotting

This was based on established methods (8, 9). Protein samples on reduced SDS-PAGE were transferred to nitrocellulose membrane filters (Schleicher & Schull, Dassel, FRG) in a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA). The transfer was done overnight at 30 V in 25 mM Tris pH 8.3 containing glycine (192 mM), SDS (0.1%), and methanol (20%). The nitrocellulose sheet was stained with Ponceau S protein stain (BDH Chemicals Ltd., Poole, England) and a photographic record was kept. The nitrocellulose was then washed in 50 mM Tris, 150 mM NaCl, 0.1% NP-40 pH 7.5 (wash buffer) containing 5% BSA for 1 h, sealed in a plastic bag with 10 ml of the same buffer containing 150 μl of antibody, and incubated for 1 h at room temperature with rocking. After incubation the nitrocellulose was thoroughly washed and sealed in a new plastic bag with 10 ml of wash buffer plus 5% BSA containing the appropriate second antibody conjugated with horseradish peroxidase (1:1,000 dilution). It was shaken for 1 h at room temperature, thoroughly rinsed, and immersed in 50 mM sodium acetate pH 5.0. Chromogenic substrate (3-amino-9-ethyl carbazole in DMSO) was added to a final concentration of 200 μg/ml and hydrogen peroxide was added to 0.01%. After color development the blots were washed in distilled water and dried.

Peptide Mapping

This was carried out as described (13). In outline, protein bands in acrylamide gels were iodinated, digested with trypsin, and the resulting peptides were first subjected to electrophoresis and then chromatographed (at right angles) on cellulose paper (Eastman Chromogram). The plates were analyzed by autoradiography on Fuji x-ray film using intensifying screens. A number of control experiments were performed to eliminate the possibility that contaminating proteins (31, 46) affected the results; maps were done of unrelated proteins such as albumin and actin (run on adjacent tracks of the same gels) and of blank pieces of gels.

Inhibition Experiments

The effect of various coagulation inhibitors on the formation of the hepatocyte extracellular matrix was studied using sparse cell cultures grown on 13-mm-diam glass coverslips. Cells were inoculated at equal density and left to attach and spread for 2 h. The coverslips were then transferred to four-well culture plates and covered with 500 μl of medium containing the appropriate inhibitors. Elaboration of an extracellular fibrillar matrix was assessed 20 h after inoculation by indirect immunofluorescent staining of fibronectin and fibrinogen.

Results

Hepatocytes Synthesize an Extracellular Matrix Containing Fibronectin and Fibrinogen

Adult rat hepatocytes seeded on various substrata (see Materials and Methods) and maintained in serum-free medium in the presence of insulin and dexamethasone were processed for immunofluorescence ~30 h after inoculation. Fibronectin immunofluorescent staining could be detected on the surface of parenchymal cells ~2 h after inoculation (Fig. 1, *a* and *b*; 2- and 3-h hepatocyte cultures, respectively). In longer-term cultures (25 h onwards) the antiserum stained an extensive fibrillar network (Fig. 1, *d*, *f*, *h–j*). Individual fibrils could often be visualized with phase contrast microscopy (Fig. 1 *c*; Fig. 1 *d* is corresponding anti-fibronectin fluorescent image). Overall three distinct classes of filaments could be distinguished depending on location and/or size. Fibers, or bundles of fibers often being several millimeters long, could be localized on top of hepatocytes, presumably anchored on dorsal cell surfaces (Fig. 1, *c* and *d*). Thinner fibers were seen on the substratum, radially emanating from the cell periphery (Fig. 1, *f* and *h*) and the entire substratum area was covered with a fine fibrillar network (Fig. 1, *f* and *h*). In addition to these thin fibers, short, thin fibrils immunoreactive with fibronectin could occasionally be detected in the cell–substratum space (Fig. 1 *i*). Overall we did not notice any difference in the fibronectin-containing matrix depending on the type of substratum used to support cell attachment.

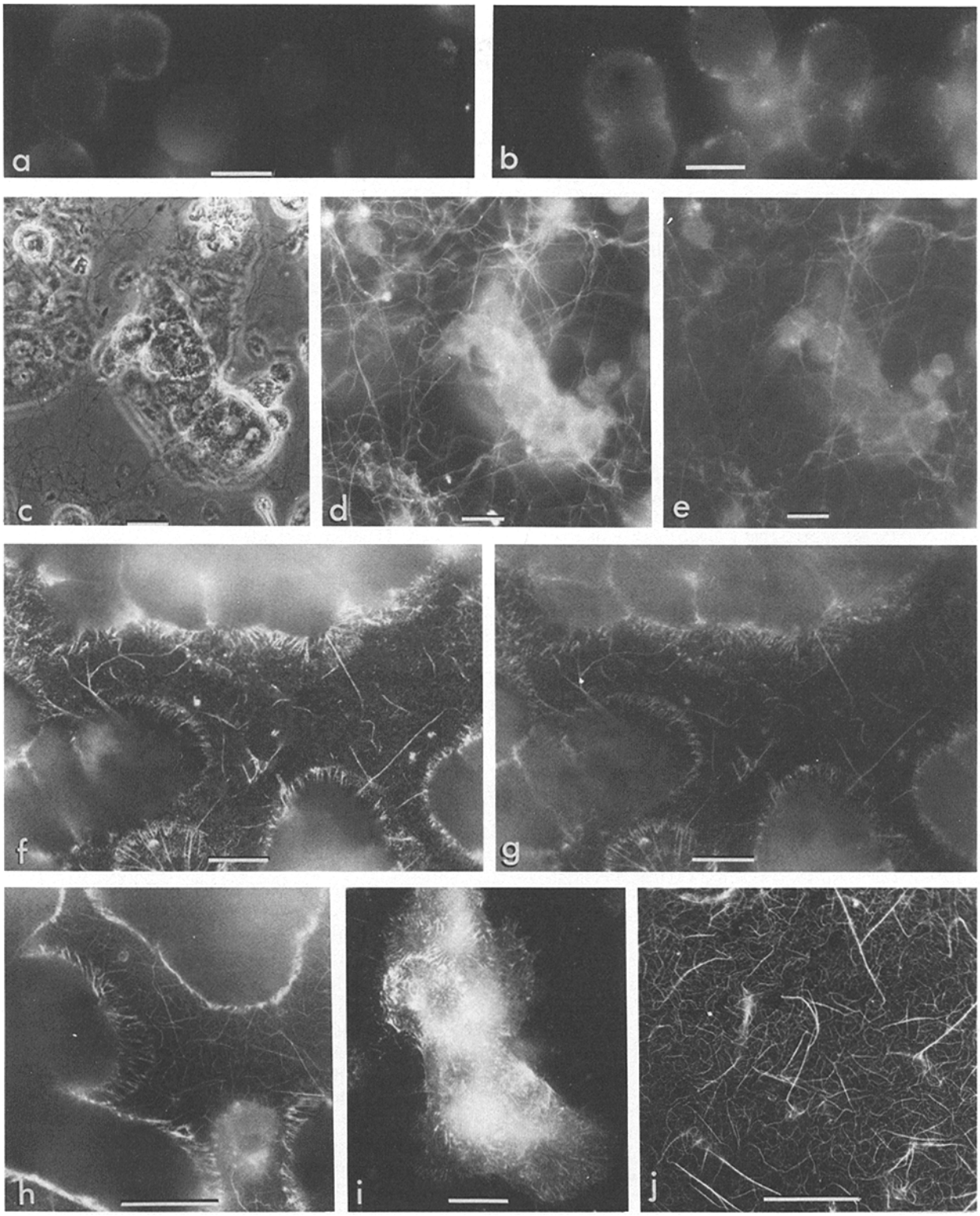

Figure 1. Hepatocytes grown either on a type IV collagen substratum in serum-free medium for 30 h (*a–i*) or cultured in the presence of serum for 2 h and thereafter maintained in serum-free medium for 30 h (*j*). (*a*) Fibronectin immunofluorescent staining of cultured hepatocytes, 2 h after inoculation. Fibronectin is just detectable on the cell surface. (*b*) Fibronectin immunofluorescence in a 3-h hepatocyte culture. Note increased fluorescence in comparison to cells in *a*. (*c*) Phase contrast image of hepatocyte culture showing extracellular matrix fibrils. The plane of focus is on dorsal cell surfaces. (*d*) Same field, fibronectin immunofluorescent staining of the extracellular matrix. (*e*) Same field, fibrinogen immunofluorescent staining. Note coincidence with fibronectin immunostaining in *d* and phase contrast image in *c*. (*f*) Fibronectin immunofluorescent staining on culture substratum. The plane of focus is on the substratum. Note pericellular filaments delineating cell periphery and finer filaments covering the substratum. (*g*) As in *f*, fibrinogen immunostaining. (*h*) Higher resolution image of fibronectin-immunoreactive fibrils on substratum intercellular spaces. (*i*) Hepatocytes stained with anti-fibronectin. The antibody penetrated the cell–substratum space and stained a fibrillar matrix. These fibrils could not be detected with anti-fibrinogen. (*j*) Matrix treated with collagenase and stained with anti-fibronectin; no degradation is seen. Bars, 20 μm.

To determine whether the matrix contains collagens or laminin, which are major components of the liver biomatrix (17, 28, 38), we used the relevant antisera in immunofluorescence experiments. Type IV collagen and laminin were localized in the cellular mass with no extracellular matrix staining being evident. The matrix did not contain any collagens type I or III either (results not shown). Lack of filamentous matrix staining with collagen antisera was observed using both formaldehyde-fixed and live cells. To confirm the absence of collagen, hepatocytes were seeded in serum-containing medium and after a 2-h incubation were maintained in serum-free medium for 30 h. The culture was then treated with perfusion-grade collagenase (0.5 mg/ml) for 30 min and upon staining with anti-fibronectin the matrix appeared indistinguishable from that in control cultures (Fig. 1 *j*).

Since liver parenchymal cells are known to synthesize fibrinogen and other components of the coagulation cascade (11) we tested the possibility that the matrix contains fibrinogen. In double-labeling experiments using fibronectin and fibrinogen antisera, fibrinogen colocalized with fibronectin along the fibrils of the extracellular matrix (Fig. 1, *d* and *e*, and *f* and *g*). This was evident on both the dorsal cell surface (Fig. 1, *d* and *e*) and the substratum-attached fibrils (Fig. 1, *f* and *g*). It was only the subcellular fibronectin-positive structures (Fig. 1 *i*) that showed virtually no immunoreactivity with the fibrinogen antibody (result not shown). The hepatocytes themselves also stained with anti-fibrinogen, but it was of interest that in occasional cell preparations containing a few nonparenchymal cells, in addition to hepatocytes, stellate cells (presumably Kupffer cells) were strongly immunoreactive with the fibrinogen antibodies. Anti-fibronectin did not react with these cells (results not shown).

In a previous study (25) a fibrillar, fibronectin-containing matrix was described and attributed to the presence of dexamethasone in the culture medium. However, in our studies we did not notice any major difference in the ability of hepatocytes to elaborate either the cell surface, or the substrate-bound matrix when the medium was depleted of dexamethasone and insulin (results not shown).

To demonstrate further that fibrinogen provides the essential component of the hepatocyte matrix, we resorted to isolation and characterization of the hepatocyte matrix and to coagulation inhibitors.

Isolation and Characterization of the Hepatocyte Extracellular Matrix

Attempts to isolate the matrix using chemical means such as ionic or nonionic detergents and denaturing agents such as urea or guanidine, alone or in combination, failed to yield an intact and cell-free preparation. However, it was feasible to physically dislodge bundles of fibers attached at their ends to dorsal cell surfaces (see Materials and Methods) and analyze these by SDS-PAGE, immunoblotting, and peptide mapping.

The main protein of the matrix had a relative molecular mass of 58K as shown by reduced SDS-PAGE and either staining with Coomassie Blue (Fig. 2 *a*) or by radioautography of metabolically radiolabeled (Fig. 2 *b*) matrix proteins. A number of other bands were also visible, the most prominent of which was of 55 kD. The 58/55-kD bands reacted in immunoblotting with anti-fibrinogen, as expected for fibrin

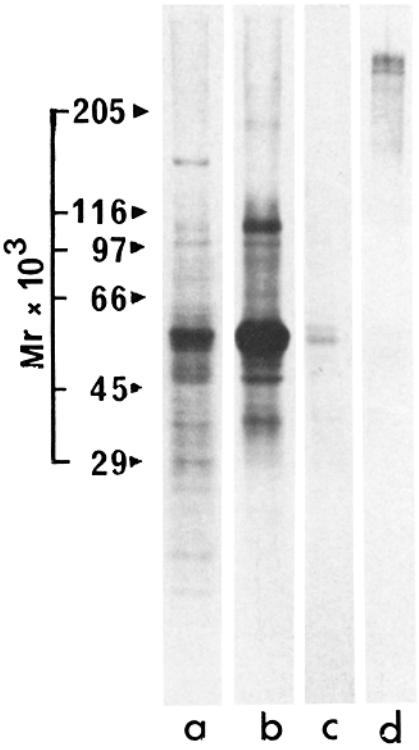

Figure 2. Analysis of the hepatocyte matrix by SDS-PAGE and immunoblotting. (*a*) Coomassie Blue–stained matrix proteins in reduced SDS-PAGE. (*b*) Fluorography of reduced matrix proteins, metabolically labeled with [^{35}S]methionine. (*c*) Matrix proteins transferred on nitrocellulose membrane and overlayed with anti-fibrinogen. (*d*) As in *c*, overlayed with anti-fibronectin.

(Fig. 2 *c*). Using anti-fibronectin the characteristic doublet at 220 kD was distinguished (Fig. 2 *d*).

The relation between the 58-kD band and the β-monomer of fibrinogen was unequivocally established by tryptic peptide maps of the matrix component and of the subunits of reference standard rat fibrinogen (Fig. 3). The 58-kD band of the hepatocyte matrix (Fig. 3, m_1) gave a similar peptide pattern after trypsin digestion, electrophoresis, and chromatography to that of the β-chain monomer of fibrinogen (Fig. 3, *b* and *e*, respectively). The 55-kD peptide (Fig. 3, m_2) could be a degradation product of the 58 kD protein (Fig. 3 *c*). On the basis of published results (29), the band of 105 kD (Fig. 2, *a* and *b*) could be γ-chain dimers; this band is weakly immunoreactive with anti-fibrinogen (Fig. 2 *c*). α-chain polymers (M_r > 400,000; reference 29) were not seen, possibly due to their low solubility in the electrophoresis sample buffer and hence exclusion from the gel.

Inhibition of Matrix Formation and Fibrinolysis

Inhibition of matrix formation was drastic with heparin and antithrombin III. Hepatocyte cultures seeded on type IV collagen were maintained in serum-free medium in the presence of antithrombin III (1 U/ml) and heparin (5 μg/ml) for 30 h and then examined for fibronectin and fibrinogen immunoreactivity. The results are shown in Fig. 4, *a* (fibronectin immunostaining) and *b* (same field, fibrinogen immunostaining). No fibrillar matrix was found either on the dorsal hepatocyte surfaces or attached to the substratum. Instead,

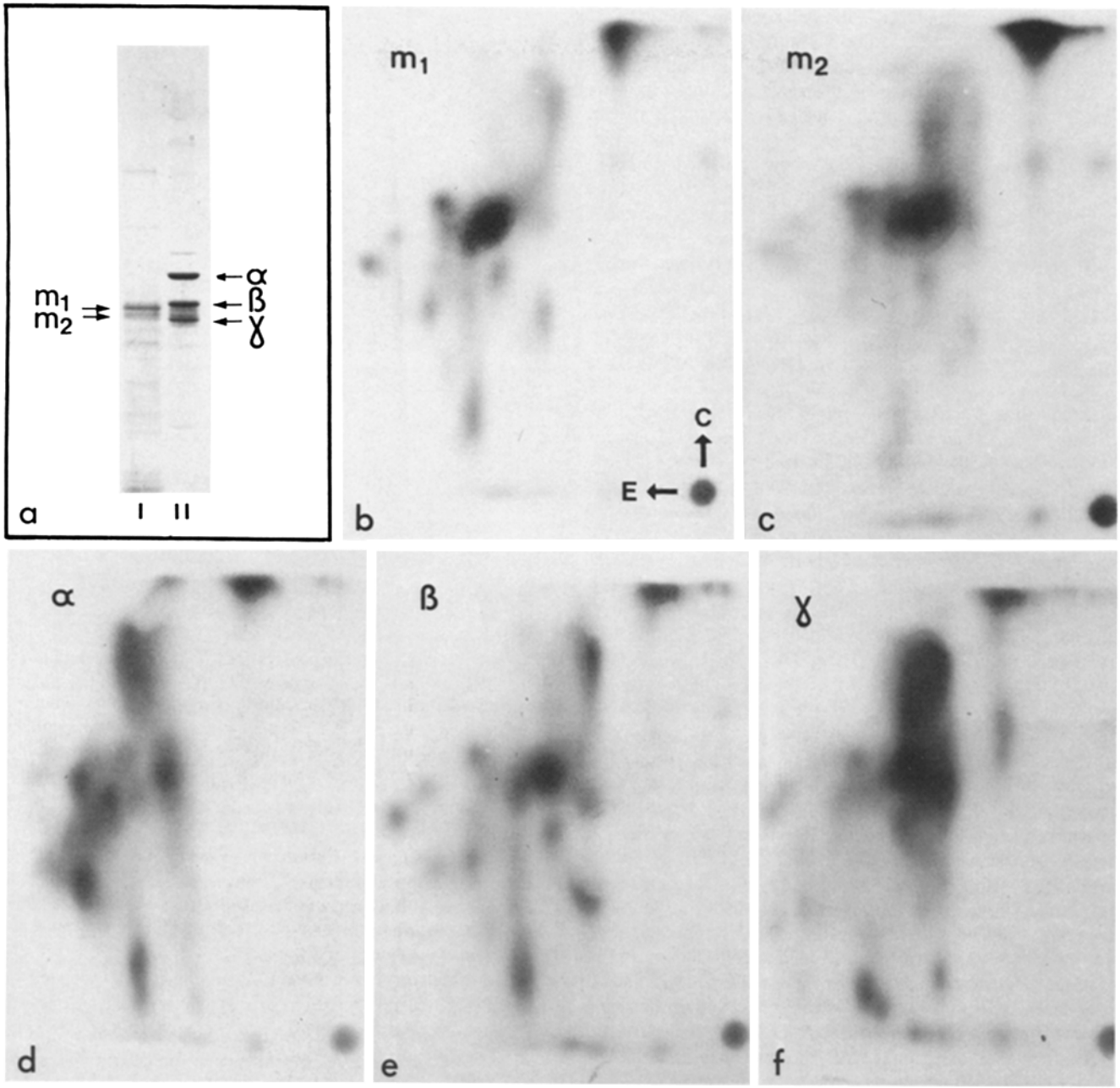

Figure 3. Tryptic peptide maps of the major proteins in the hepatocyte extracellular matrix and of standard fibrinogen chains. (*a*) Hepatocyte matrix (lane *I*) run alongside rat fibrinogen (lane *II*) in SDS-PAGE. The positions of the α-, β-, and γ-fibrinogen chains and of the major matrix proteins are indicated. These bands were cut off, iodinated, and trypsinized, and the resulting peptides were subjected to electrophoresis and then chromatographed at right angles. (*b*) Peptide map of the 58-kD matrix protein. Arrows indicate direction of electrophoresis (*E*) and chromatography (*C*). (*c*) Peptide map of the 55-kD peptide. (*d*) Peptide map of the α-fibrinogen chain. (*e*) Peptide map of the β-fibrinogen chain. (*f*) Peptide map of the γ-fibrinogen chain.

a punctate pericellular fluorescence was observed when the fibronectin antiserum was used (Fig. 4 *a*). These fibronectin-positive sites were mostly devoid of fibrinogen (Fig. 4 *b*). Fibronectin failed to elaborate a matrix on its own. This inhibition took place in the presence of heparin (0.5–5 μg/ml) alone whereas antithrombin (1 U/ml) in the absence of heparin had a markedly reduced effect (not shown).

The inhibitory action of heparin was analyzed further using heparin oligosaccharide fragments of different chain lengths and with high or low affinity for antithrombin III (24, 48). Dermatan sulfate and hirudin were also used. High-affinity heparin molecules with 18 or more monosaccharides potentiate inactivation of thrombin by antithrombin III (24). Hirudin (26, 27) and dermatan sulfate (33, 35, 49) can also inhibit thrombin. Dermatan sulfate exerts its inhibitory action by activating heparin cofactor II which, in turn, inactivates thrombin (33, 49). The effects of these inhibitors on the formation of the hepatocyte matrix are summarized in Table I. High-affinity octadecasaccharide (and bigger) heparin molecules inhibited the formation of the matrix at very low

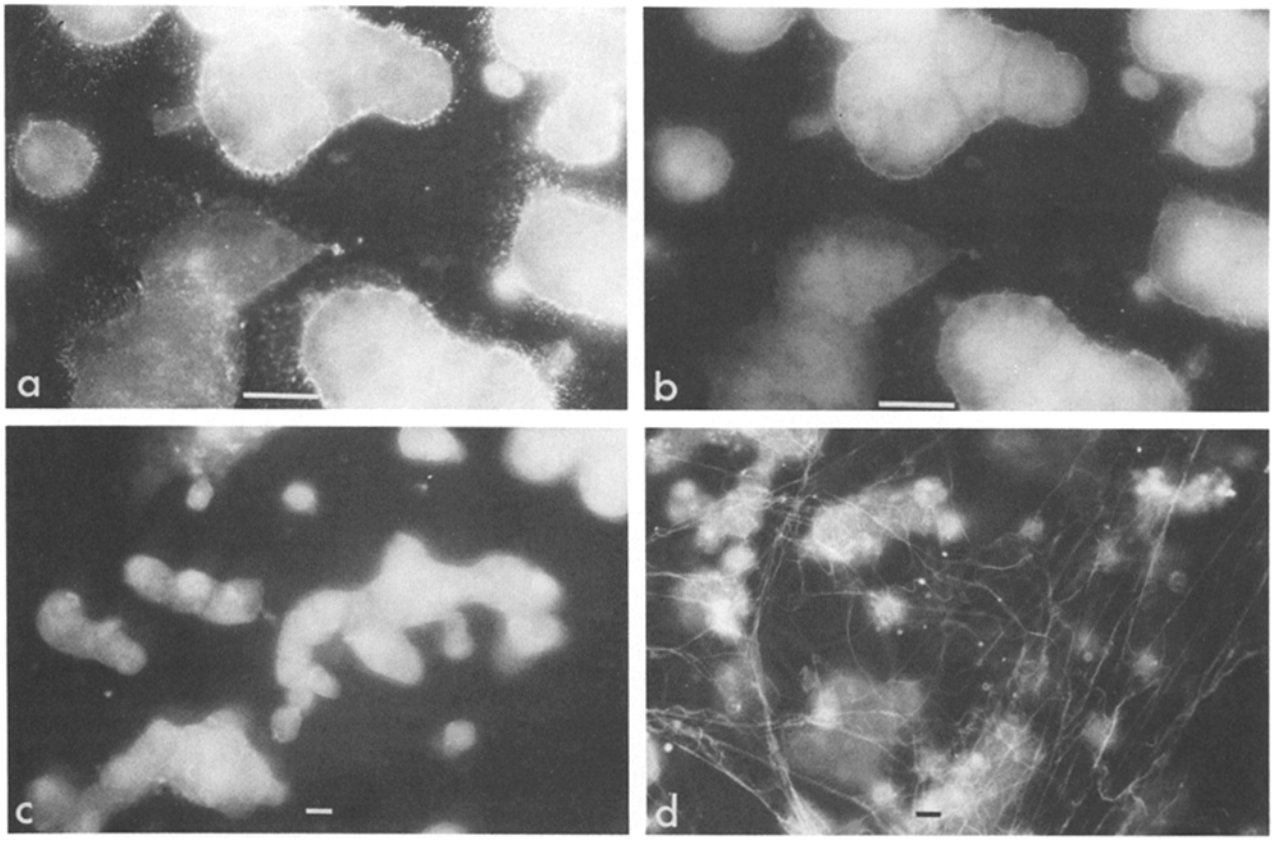

Figure 4. Immunofluorescent staining of hepatocytes maintained in culture for 30 h. (*a*) Fibronectin staining of hepatocytes on type IV collagen maintained in serum-free medium in the presence of antithrombin III and heparin. No matrix was formed. (*b*) Same field, fibrinogen staining. (*c*) Fibronectin staining of hepatocytes maintained in serum-containing medium. Surface fibrils are reduced in number. (*d*) Control experiment: hepatocytes were inoculated in the presence of serum for 2 h and thereafter maintained in serum-free medium. Fibronectin immunostaining. Bars, 20 μm.

concentrations (50–100 ng/ml), whereas heparin oligosaccharides with low affinity for antithrombin III were not effective inhibitors. Furthermore, tetradecasaccharides or smaller high-affinity fragments failed to inhibit fibrin deposition at concentrations up to 1 μg/ml. Hirudin and dermatan sulfate inhibited formation of the matrix (Table I).

Another anticoagulant tested was warfarin, a drug administered to patients with thrombotic diathesis, which is purported to inhibit enzymes that catalyze the reduction of vitamin K to its active hydroquinone form (15, 43). Vitamin K functions as a cofactor for vitamin K–dependent carboxylase which activates coagulation zymogens (43, 44). When hepatocytes were maintained in serum-free medium containing warfarin (50–500 μM) the amount of matrix associated with dorsal cell surfaces was reduced, but no change could be observed in the substratum-bound matrix. Visually, the effect was similar to that of maintaining the cultures in serum-containing medium (see below).

Addition of plasmin (0.3 U/ml), the main enzyme in the fibrinolytic pathway, in 40-h hepatocyte cultures, resulted in rapid degradation of the matrix and in cell rounding. In separate experiments we found that glass-adsorbed fibrinogen did not support hepatocyte adhesion in the presence of 20 μM cycloheximide that was added to prevent synthesis and secretion of fibronectin and other spreading factors. Since plasmin treatment finally detached cells, presumably some proteolysis of adhesive factors such as fibronectin occurred in addition to fibrin degradation.

Effect of Serum

When hepatocytes were seeded in the presence of serum and the culture medium was replaced 2 h after inoculation with serum-free medium, the appearance of the matrix was indistinguishable after 25–52 h in culture from that of cells seeded on type IV collagen, fibronectin, or laminin in serum-free medium. However, we noticed that if hepatocytes were maintained in serum-containing medium, the amount of fibrillar matrix was significantly decreased. Fig. 4, *c* and *d* illustrate hepatocyte cultures that were inoculated in the presence of serum. After a 2-h incubation cells were maintained either in serum-containing (Fig. 4 *c*) or in serum-free (Fig. 4 *d*) medium for 25 h. Fibronectin immunostaining showed that inclusion of serum resulted in reduced matrix accumulation on dorsal cell surfaces (Fig. 4, *c* and *d*). No effect on the substrate matrix could, however, be distinguished. Also a certain variation of this serum inhibition on matrix accumulation was observed among different serum batches.

Discussion

We have shown that hepatocytes in serum-free primary culture synthesize a fibronectin-containing fibrin clot. Although

Table I. Inhibition of Fibrin Coagulation in Hepatocyte Cultures by Glycosaminoglycans and Hirudin

Inhibitor	Inhibited protease	Concentration	Inhibition of matrix formation‖
		glycosamino-glycans/μg/ml hirudin/U/ml	
Heparin HA 40–50*‡	Thrombin	0.05–2	+
Heparin LA 40–50*		0.05–2	−
Heparin HA 18–24*‡	Thrombin	0.05	−
		0.1–2	+
Heparin LA 18–24*		0.05–1	−
		2	±
Heparin HA 10–14*‡	Factor Xa	0.05–1	−
		2	±
Heparin LA 10–14*		0.05–2	−
Dermatan sulfate§	Thrombin	1	−
		10	±
		20	+
Hirudin	Thrombin	2–40	+

Inhibitors were added in freshly seeded hepatocyte cultures maintained in serum-free medium (see Materials and Methods). The cultures were immunostained for fibronectin and fibrinogen 20 h after inoculation and the presence or absence of extracellular matrix was noted.

* Heparin oligosaccharides isolated by partial nitrous acid cleavage of heparin, affinity chromatography on antithrombin III-Sepharose, and gel filtration. *HA* and *LA*, high and low affinity for antithrombin III, respectively. Numerals indicate number of monosaccharide residues in the saccharide sequences.

‡ Activates antithrombin III.

§ Activates heparin cofactor II.

‖ Inhibition of matrix formation is indicated by +, whereas − signifies presence of fibrillar matrix at equal density to that in control cultures.

fibrillar extracellular structures in hepatocyte cultures have been described in earlier studies using scanning electron microscopy (52) and fibronectin immunofluorescent staining (25, 50), results on the molecular composition of the matrix have not, to our knowledge, been published before. In one of these studies (25) the formation of this matrix was thought to be induced by the synthetic glucocorticoid dexamethasone. Our observations find this claim unwarranted. We stress, however, the limitation of immunofluorescence in conclusively establishing quantitative differences. Hepatocytes are known to secrete fibrinogen and fibronectin (11, 34, 42, 45, 50) and this secretion is known to increase upon addition of dexamethasone (1, 37), as is the secretion of other acute phase reactants (3, 4, 34).

The type of exogenous matrix substratum used did not seem to affect the formation of the fibrin–fibronectin matrix and elaboration of this matrix was neither necessary nor sufficient for initial cell attachment and spreading since these phenomena proceeded in the absence of protein synthesis but required the presence of an exogenous matrix substratum for initiation (6). Fibrinogen itself did not support hepatocyte adhesion, but fibronectin fibrils were found on the underside of spread cells suggesting an involvement of endogenous matrix-associated fibronectin in cell adhesion of established cultures. This requirement for fibronectin in cell adhesion to fibrinogen and fibrin was demonstrated in earlier studies (16), although recently fibronectin was shown to be required for migration of cells (fibroblasts) into plasma clots but not for cell adhesion and spreading (21).

Polymerized fibrin is generated in vivo by the action of the serine protease thrombin on soluble fibrinogen (for reviews on blood coagulation see references 12, 18). Thrombin is the final product in a cascade of zymogen activations into serine proteases; each activated protease catalyzes the next reaction of the series. Most of the proteins in the coagulation cascade are made in the liver parenchyma (11, 54, 55) and our results showing the formation of a fibrin clot in isolated hepatocyte cultures emphasize the role of the hepatocyte in coagulation. Nonparenchymal cells were scarcely observed in our cultures and the matrix was seen to be closely associated with the hepatocyte surface.

At this stage we cannot define the factor(s) involved in the initiation of the clotting mechanism in hepatocyte cultures. The hepatocyte apparently produces proteins required for blood coagulation according to the intrinsic as well as the extrinsic pathway of activation (11, 54, 55). However, thromboplastin, the specific trigger component of the extrinsic pathway, was not detected in significant amounts in our cell cultures (assay performed by Dr. Hans Prydz, University of Oslo, Norway). In our system, formation of the fibrin network was prevented by agents that inactivate, or promote the inactivation of, thrombin. Heparin oligosaccharides with high affinity for antithrombin III thus accelerate the inhibitory effect of antithrombin III on this protease (7), provided that the saccharide chain equals or exceeds 18 monosaccharide units; shorter fragments selectively promote the inactivation of factor Xa (24). Moreover, thrombin is inhibited by heparin cofactor II, and this reaction is stimulated not only by heparin (with high or low affinity for antithrombin III) but also by dermatan sulfate, another sulfated glycosaminoglycan with anticoagulant properties (32, 33, 35, 49). Finally, thrombin is directly and specifically inactivated by hirudin (26, 27). The inhibitory effects of the various saccharides, and of hirudin, on the generation of fibrin by the hepatocytes strongly suggest that this process occurs by specific cleavage of fibrinogen by thrombin. The significant, albeit weaker, effect of smaller heparin oligosaccharides with high affinity for antithrombin is also compatible with the involvement of factor Xa. The two reactions catalyzed by factor Xa and thrombin conclude the coagulation process (and are common to the extrinsic and intrinsic pathways). These conclusions imply that both antithrombin III and heparin cofactor II are synthesized by the liver parenchyma cells, in accordance with results of other studies (22).

An important deduction from our observations is that the hepatocyte heparan sulfate (19, 36) is either incapable of, or insufficient in quantity to exert inhibition of clotting. This may appear to contradict reports on the anticoagulant properties of heparan sulfates from liver (49) and inhibition experiments with purified rat liver heparan sulfate will be needed to clarify the issue. Heparan sulfates from other tissues, especially endothelium, are known inhibitors of coagulation (33, 41, 47).

Significant fibrinolysis did not appear to take place in hepatocyte cultures, as expected, since hepatocytes produce plasminogen activator but not plasminogen (53), the inactive precursor of plasmin. The partial inhibition of matrix accumulation in cultures maintained in serum could be due to the presence of plasminogen in the serum. An alternative possibility is that antithrombin is present in serum in sufficient concentration to inhibit fibrin formation even in the absence of added polysaccharide. The incomplete inhibition

by warfarin is possibly due to the existence of an alternative pathway for vitamin K reduction which is not affected by coumarin drugs (51).

The liver is of fundamental importance in hemostasis and impaired function could precipitate severe abnormalities in coagulation, such as hemorrhage or disseminated intravascular coagulation (10, 11). The hepatocyte in culture may offer a simple system to study the regulation of coagulation and to assess the use of factors such as activators or inhibitors to modulate clotting.

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