Maintenance of Differentiated Phenotype of Cultured Rat Hepatic Lipocytes by Basement Membrane Matrix*

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This study examined the role of extracellular matrix in regulating matrix phenotype of hepatic lipocytes, the major source of matrix in liver. Lipocytes (Ito, stellate, or fat-storing cells) were purified from normal rat liver and established in primary culture on either uncoated plastic, plastic coated with individual matrix proteins, or a "complete" gel matrix, a basement membrane-like matrix derived from the Engelbreth-Holm-Swarm (EHS) murine tumor. The ultrastructure of lipocytes cultured on the gel matrix resembled that of cells in normal liver, whereas lipocytes on plastic had dispersed nuclear chromatin and expanded rough endoplasmic reticulum, consistent with active proliferation and secretion. Lipocytes on the gel matrix exhibited no proliferative activity; cells maintained on plastic proliferated and produced type I collagen predominantly. Total collagen secretion by lipocytes on the gel matrix was 29% of that of cells on plastic, and consisted of type III collagen only. This difference extended to proteoglycan production, which was less than 5% of the amount produced by cells in conventional culture on plastic. The effects of the EHS gel were not reproduced by the individual components of the gel (laminin, type IV collagen, and heparan sulfate proteoglycan) or by a type I collagen gel. They were also reversible upon transfer of the cells to conventional culture. In contrast to lipocytes, collagen synthesis by hepatocytes was similar whether cultured on EHS gel or on plastic. These results show that the extracellular matrix can modulate matrix protein production by lipocytes and imply that, in early hepatic inflammation, changes in the hepatic subendothelial matrix may underlie stimulation of lipocyte matrix production and progression of the fibrotic process.

Efforts to clarify the pathophysiology of tissue fibrosis are focused increasingly on how fibroblasts, smooth muscle cells, or pericytes are stimulated to proliferate and secrete connective tissue matrix. Pericytes, in particular, are defined by their intimate relationship to capillary endothelium and their smooth muscle cell markers (1, 2) and are distinguished from smooth muscle cells by their fibroblast-like morphology. In liver, pericytes are represented by lipocytes (Ito, stellate, or fat-storing cells), where they are also identified by their ability to store hepatic retinoids (3). Lung "fibroblasts" (4, 5) and kidney mesangial cells (6) are other examples of organ-specific pericytes that may be involved in fibrosis. Studies in liver suggest that lipocytes under appropriate stimulation, modulate from quiescent "contractile" cells to activated "synthetic" cells, acquiring morphologic changes that mark them as "myofibroblasts" (7, 8).

The mechanism of pericyte activation represents a major issue with respect to the pathogenesis of inflammation and to potential points of therapeutic intervention in fibrotic disease. Although cell culture offers an attractive direct approach to this area, it requires a source of resting (unstimulated) pericytes. The liver provides this, in that enzymatic dispersion of the tissue yields a pure population of lipocytes in quantities sufficient for mass primary culture (9). Studies of this system are beginning to provide information on the role of soluble factors that may regulate proliferation and matrix expression (10, 11). A regulatory role of extracellular matrix also is possibly of major importance: lipocytes are within the subendothelial matrix and thus are positioned both to modulate its composition by new synthesis and to be affected by it. The latter question has not been studied, although matrix is known to regulate the phenotype of various mesenchymal cells (12, 13). In a recent report, Davis and co-workers (14) used lipocytes in serial subculture, studying their response to individual matrix proteins as substrata. Although the work documents a modulating effect of matrix on multiply passaged cells, it does not directly address the question of lipocyte activation in vivo because cells passaged for several generations are highly proliferative and thus already activated.

The current study describes the role of complete matrix in maintaining the differentiated phenotype of freshly isolated lipocytes in primary culture. On this basement membrane-like substratum, the cells maintain a rounded shape, minimal proliferation, and little matrix synthesis. This is in striking contrast to cells on collagen or plastic, which display an activated phenotype. This effect of extracellular matrix on collagen production was seen only in lipocytes and not in hepatocytes cultured under similar conditions. Given that lipocytes are major matrix producers (15–19), the data suggest that changes in lipocyte matrix production induced by an altered extracellular matrix may perpetuate an activated state.
**LIVER CELL CULTURE—**Hepatic lipocytes were isolated from normal male Sprague-Dawley rats as described previously (9). In brief, the liver was dispersed by perfusion with Percoll and collagenase, which destroys the hepatocytes. The resulting non-parenchymal cell suspension was filtered through cotton gauze and washed three times in Eagle's minimal essential medium containing D-Nase, and then centrifuged through a discontinuous gradient of 6, 8, 12, and 15% arabinogalactan (Stractan; Larex International Corp., Tacoma, WA). Purified lipocytes were recovered from the interface between 6% Stractan and medium, washed in Eagle's minimal essential medium, and plated in Medium 199 (20) supplemented with 10% each calf and horse serum, insulin (4 milliunits/ml), ascorbate (50 µg/ml), penicillin (100 units/ml), corticosterone (10⁻⁶ M), and HEPES buffer (10 mM) on either uncoated tissue plastic (Lusk; Miles Scientific, Naperville, IL) or plastic coated with EHS matrix (see below) at a density of 2-4 x 10⁶ cells/cm². Purity was assessed by the use of specific fluorescent markers for lipocytes, Kupffer cells, and sinusoidal endothelial cells (9). Cultures were incubated at 37 °C in a humidified atmosphere containing 95% O₂, 5% CO₂, and the medium was changed every 24 h.

Hepatocytes were prepared from collagenase-dispersed liver and purified by centrifugal elutriation as described previously (21). Isolated cells were cultured on 35-mm plastic dishes coated with acid-soluble collagen (22), or on EHS. The EHS sarcoma was passaged in Swiss-Webster mice and EHS gel prepared as described previously (22), except that the final 2 M urea extract was dialyzed against phosphate-buffered saline, pH 7.4. The liquid EHS was pipetted at 4 °C into 35-mm culture dishes, 0.6 ml/plate, and allowed to gel at 37 °C for 24 h prior to use. It contained approximately 96% laminin, 6% type IV collagen, and 4% heparan sulfate proteoglycan (22). Cells cultured on the gel were released with Dispase (Collaborative Research, Cambridge, MA), 2 ml/plate at 37 °C for 1 h, and recovered by centrifugation. Gels of type I collagen were prepared from rat tail tendon exactly as described by Belows et al. (23) with a volume of 0.6 ml/35-mm plate. Laminin, type IV collagen, and heparan sulfate proteoglycan used for coating micro-wells were prepared from EHS tumor as described previously (22). Plates with 24 wells were incubated overnight at room temperature with either laminin (20 µg/ml), type IV collagen (1 µg/ml), or heparin sulfate proteoglycan (250 µg/ml) and suspended in either plating medium (for laminin or heparin sulfate proteoglycan) or 2% BSA, 2 mM dithiothreitol, 8 mM EDTA (for type IV collagen). Wells were washed twice with phosphate-buffered saline prior to cell plating.

**Matrix Synthesis—**Lipocytes (7 days old) or hepatocytes (2 days old) in primary culture, were plated and maintained in a proline-free modification of Medium 199 (to avoid dilution of the label by unlabelled amino acid) containing 20% serum and then labeled either for 1 h in medium containing 500 pCi/ml [2,3,4,5-³H]proline, or for 1 h in medium containing 50 pCi/ml [2,3,4,5-³H]proline, [³H]proline, or [³H]glycine, respectively (25).

DNA Synthesis—Cultures of lipocytes on either EHS gel or plastic were maintained for 12 days in medium containing 20% serum prior to labeling with [³H]thymidine. Cells were washed twice with phosphate-buffered saline and then incubated for 2 h at 37 °C in serum-free Medium 199 containing 10 µCi/ml [³H]thymidine with or without 10 mM hydroxyurea (29). The medium was discarded, and the cell layer was washed twice with cold phosphate-buffered saline, inoculated for 15 min at 4 °C in 10% trichloroacetic acid, washed once with absolute ethanol at 4 °C, and then incubated for 30 min with 30% perchloric acid at 0 °C. The precipitate was dissolved in 1 N KOH, and counted in liquid scintillant. Cultures were incubated at 37 °C in Medium 199 (with or without 10 µM hydroxyurea) for 24 h. The medium was discarded, and the cell layer was washed twice with cold phosphate-buffered saline, inoculated for 15 min at 4 °C in 10% trichloroacetic acid, washed once with absolute ethanol at 4 °C, and then incubated for 30 min with 30% perchloric acid at 0 °C. The precipitate was dissolved in 1 N KOH, and counted in liquid scintillant. The data (cell layer plus medium) were normalized for DNA content (24).
postfixed in 1% OsO₄ for 15 min at 4 °C, rinsed in water, and placed in uranyl acetate (2% in water) overnight (30). The cells were either dehydrated in an ethanol series in the plates or removed from the plates in propylene oxide, pelleted, and embedded. Sections were cut, stained with 2% uranyl acetate and Reynolds lead citrate, and photographed in a JEOL JEM-100 CX II electron microscope.

**Statistical Methods**—Results were analyzed by either paired or unpaired Student's t test or Mann-Whitney test.

**RESULTS**

**Matrix-mediated Changes in Morphology**—Lipocytes cultured on uncoated plastic progressively spread and flattened (Fig. 1A). In contrast, cells plated on EHS gel formed clusters connected by a filamentous network extending from the cells (Fig. 1B). At the ultrastructural level, the cells in conventional culture on plastic exhibited marked hypertrophy of rough endoplasmic reticulum and Golgi apparatus (Fig. 2). They also had extensive microfilaments (16) and large nuclei with little heterochromatin and prominent nucleoli (Fig. 2). Lipocytes on the EHS substratum were compact and embedded within the gel (Fig. 3). Rough endoplasmic reticulum and the Golgi apparatus were minimally developed and nuclear chromatin was condensed. All cells appeared viable, with virtually no dead cells trapped within the gel. This appearance was similar to that of freshly isolated lipocytes (16) or cells in situ (31) but in marked contrast to the lipocytes cultured on plastic.

**Modulation of Matrix Synthesis by Lipocytes**—Cells cultured for 6–7 days on EHS gel, then labeled for 24 h with [³H]proline, incorporated significantly less radioactivity into collagen than did parallel cultures maintained on plastic for the same period (Table I). Both collagen and non-collagen protein synthesis were 35% of that produced by conventional cultures. In separate experiments under similar labeling conditions (data not shown), the intracellular free proline pools in cells on plastic and on EHS gel were not significantly different, suggesting that the observed differences in rates of collagen synthesis were not due to a specific defect in amino acid uptake. To examine whether proteolysis of newly synthesized collagen was playing a role, we labeled cells for 1 h only (see "Materials and Methods") or in the presence of 20% serum (which inhibits extracellular proteases) with results similar to those shown in Table I (data not included). Therefore, the matrix-related changes in collagen production appear to reflect differing rates of synthesis.

Type-specific collagen formation was examined by SDS gel electrophoresis and autoradiography of labeled material precipitated from lipocyte medium with ammonium sulfate. Cells cultured on plastic produced type I collagen predominantly, as shown previously (16). In contrast, lipocytes on the EHS gel elaborated almost exclusively type III collagen (Fig. 4). The difference in type-specific synthesis was clearest when reduced and unreduced samples were compared prior to peptidase digestion (lanes A and B versus A' and B'). The collagenous nature of radiolabeled bands was confirmed by their susceptibility to bacterial collagenase (lanes C and C').

Studies of proteoglycan synthesis demonstrated that the effects of the culture substratum involved matrix production broadly. As shown in Fig. 5, modulation of proteoglycan synthesis by the substratum was profound. Cells on EHS gel synthesized less than 5% of the glycosaminoglycans produced by cells on plastic (predominantly dermatan and chondroitin sulfates).

**Selectivity of Matrix-induced Changes**—Hepatocyte cultures were used to examine effects of the substratum on collagen synthesis by an epithelial cell type. 2-day-old cultures were incubated with [³H]proline, as described under "Materials and Methods." This early time point was chosen to ensure that measurements reflected collagen synthesis by hepatocytes and not by a subpopulation of lipocytes which may emerge in older cultures (32). For hepatocytes cultured on EHS and on plastic, rates of collagen synthesis did not differ significantly. Total protein synthesis also was unaffected by the culture substratum (Table II).

**Effects of EHS on Lipocytes Are Reversible and Matrix-specific**—Freshly isolated lipocytes, maintained on EHS gel for 7 days, were released with Dispase (which liquifies the gel) and replated onto uncoated plastic tissue culture dishes. Within 3 days, the replated cells had spread and acquired an appearance similar to those plated initially on plastic (data not shown). Their collagen and protein synthesis also in-
was: plastic, 67% cell layer, 33% medium; EHS gel, 83% cell layer, 17% medium.

were also compared to cells on the individual components of the EHS gel (Fig. 7, C-E), and on a type I collagen gel (Fig. 7F). None of the components or the collagen gel reproduced the morphology of cells on the EHS gel. Furthermore, total collagen and protein synthesis by cells on type I collagen gel were no different than for cells on plastic (data not shown), suggesting that the modulating effect of the EHS gel was due to its specific composition and not to its physical properties as a gel.

The Effect of Matrix on Lipocyte Proliferation—Studies were performed with cells maintained for 12 days in culture (cells begin to proliferate only after a lag period of 8-10 days) (9, 33). Lipocytes on an EHS gel substratum were quiescent, whereas cells from the same isolate cultured on either uncoated plastic or plastic coated with laminin or type IV collagen exhibited similar levels of DNA synthetic activity (Fig. 8). Reliable data for cells on heparan sulfate proteoglycan could not be obtained because of the poor plating efficiency of lipocytes on this substratum. For cells on uncoated plastic, thymidine incorporation into DNA was inhibited 85-90% by

Table I

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Peptide-bound amino acid*</th>
<th>Collagen synthesis†</th>
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<tbody>
<tr>
<td></td>
<td>[3H]Hydroxyproline [3H]Proline</td>
<td></td>
</tr>
<tr>
<td>Plastic (n = 3)</td>
<td>40.1 ± 2.4 447.7 ± 63.7</td>
<td>3.95</td>
</tr>
<tr>
<td>EHS gel</td>
<td>11.7 ± 3.3  142.2 ± 31.5</td>
<td>3.61</td>
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The values shown are mean ± S. E. for three separate experiments of three replicates each. Total data for [3H]hydroxyproline and [3H]proline represent the sum of incorporated disintegrations/min in cell plus medium layers. Distribution of disintegrations/min [3H]hydroxyproline was: plastic, 46% cell layer, 54% medium; EHS gel, 66% cell layer, 34% medium. Distribution of disintegrations/min [3H]proline was: plastic, 67% cell layer, 33% medium; EHS gel, 83% cell layer, 17% medium.

† The values shown are mean ± S. E. for three separate experiments of three replicates each. Total data for [3H]hydroxyproline and [3H]proline represent the sum of incorporated disintegrations/min in cell plus medium layers. Distribution of disintegrations/min [3H]hydroxyproline was: plastic, 46% cell layer, 54% medium; EHS gel, 66% cell layer, 34% medium. Distribution of disintegrations/min [3H]proline was: plastic, 67% cell layer, 33% medium; EHS gel, 83% cell layer, 17% medium.

* Calculated using a modification of the formula of Diegelmann, as described previously (25).

† p < 0.05 compared with cells on plastic.
**TABLE II**

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Peptide-bound amino acid$^b$</th>
<th>Collagen synthesis$^a$</th>
</tr>
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<tbody>
<tr>
<td>Plastic (n = 3)</td>
<td>$10.3 \pm 2.6$</td>
<td>$1148.6 \pm 199.2$</td>
</tr>
<tr>
<td>EHS gel (n = 3)</td>
<td>$7.7 \pm 1.4^c$</td>
<td>$628.8 \pm 140.5$</td>
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</table>

$^a$ Values shown are mean ± S. E. for three separate experiments of three replicates each. Total data for $[^3]H$hydroxyproline and $[^3]H$proline represent the sum of incorporated disintegrations/min in cell plus medium. Distribution of disintegrations/min $[^3]H$hydroxyproline was: plastic, 59% cell layer, 41% medium; EHS gel, 68% cell layer, 32% medium. Distribution of disintegrations/min $[^3]H$proline was: plastic, 56% cell layer, 44% medium; EHS gel, 60% cell layer, 40% medium.

$^b$ Calculated using a modification of the formula of Diegelmann, as described previously (27).

$^c$ p, not significant compared with cells on plastic.

**DISCUSSION**

Lipocytes, as liver pericytes, are ideally positioned to both direct and respond to matrix alterations in the subendothelial space. The cells extend numerous processes that encircle the sinusoid (3). The presence of cytoplasmic desmin both in situ (34, 35) and following isolation (9, 36), as well as cGMP kinase (2), suggests that lipocytes are related to smooth muscle cells. Following liver injury, their "activation" to myofibroblast-like cells has been demonstrated: vitamin A deposits are lost, cell elongation occurs, rough endoplasmic reticulum hypertrophies, and pericellular collagen appears (8, 37, 38). Desmin, however, remains demonstrable in these cells (35). Studies in animals have also shown proliferation of lipocytes/myofibroblasts following hepatic injury (39, 40).

**FIG. 6. Reversibility of extracellular matrix-induced changes on lipocyte collagen synthesis.** Lipocyte isolates were divided and plated on either uncoated tissue culture plastic or EHS gel and grown for 7 days. Collagen (open bars) and total protein synthesis (stippled bars) were assayed as described under "Materials and Methods." At this point, cells cultured initially on EHS gel were released with Dispase, replated on uncoated plastic, and maintained for an additional 7 days, after which measurement of total protein synthesis was repeated. The data are expressed as peptide-bound radioactivity in hydroxyproline or proline, normalized for DNA content. Results from cells maintained on plastic are set at 100% for comparison with cells maintained on EHS gel. The data represent mean values and ranges obtained in three separate experiments, each of which was performed in duplicate. Error bars represent range.

hydroxyurea, indicating that synthesis was directed to replication and not repair.

**FIG. 7. Phase-contrast micrographs of lipocytes on plastic, EHS gel, individual components of EHS gel, and type I collagen gel.** The culture substrata are: A, tissue culture plastic; B, EHS gel matrix; C, type IV collagen; D, heparan sulfate proteoglycan; E, laminin; or F, type I collagen gel. On all matrices except EHS gel, cells exhibit spreading, with prominent nuclei (arrows) and nucleoli, whereas cells on EHS gel form dense clusters with cytoplasmic-processed connecting clusters. All cultures were maintained in primary culture for 7 days in the presence of 20% serum. Bar equals 50 μm.

**FIG. 8. Replicative DNA synthesis by lipocytes cultured on plastic, EHS gel, or EHS gel components.** Lipocytes were maintained for 12 days in primary culture on either uncoated tissue culture plastic, EHS gel, type IV collagen, or laminin in the presence of 20% serum and then incubated for 3 h in medium containing $[^3]H$thymidine. Values for incorporated $[^3]H$ were normalized for cell number. Mean values for three experiments in disintegrations/min/10^6 cells for 3 h were: plastic, 1627.5; EHS gel, 320.1; type IV collagen, 2199.0; and laminin, 1903.2. Results shown are the mean values and ranges of proliferative activity expressed as percent of that on plastic (control) for three separate experiments, each of which was performed in triplicate. Error bars represent range; *p < 0.02.

$^2$ J. Boyles, unpublished observations.
These two states, "normal" and "activated," may be modeled by lipocytes cultured on EHS gel and plastic, respectively. The principal constituents of the EHS gel, laminin, type IV collagen, and heparan sulfate proteoglycan (22), are present within the subendothelial space of the normal liver, although comparisons at the level of the detailed molecular structure of the gel are not possible. The biologic effects of EHS gel further support its being a normal substratum. On EHS gel, lipocytes are compact and nonproliferative in the same medium (20% serum-containing) that supports proliferation and collagen secretion by cells on plastic. Ultrastructurally, they appear quiescent, with a paucity of rough endoplasmic reticulum and Golgi complexes. The switch in collagen type from production of type III by cells on EHS to type I by lipocytes on plastic also is consistent with activation of the cells on plastic. Of the total collagen in normal liver, approximately one-half is type III (41, 42), and some is distributed, as are proteoglycans within the subendothelial space of the normal liver, although depleted by lipocytes cultured on EHS gel and plastic, respectively. The principal constituents of the EHS gel, laminin, type IV collagen, and heparan sulfate proteoglycan (22), are present within the subendothelial space of the normal liver, although comparisons at the level of the detailed molecular structure of the gel are not possible. The biologic effects of EHS gel further support its being a normal substratum. On EHS gel, lipocytes are compact and nonproliferative in the same medium (20% serum-containing) that supports proliferation and collagen secretion by cells on plastic. Ultrastructurally, they appear quiescent, with a paucity of rough endoplasmic reticulum and Golgi complexes. The switch in collagen type from production of type III by cells on EHS to type I by lipocytes on plastic also is consistent with activation of the cells on plastic. Of the total collagen in normal liver, approximately one-half is type III (41, 42), and some is distributed, as are proteoglycans within the subendothelial space of the normal liver, although depleted by lipocytes cultured on EHS gel and plastic, respectively. The mechanism of lipocyte activation and collagen type-switching in vivo remains to be determined. The primary event may be the release of soluble factors by Kupffer cells (10, 13), stimulating production of type I collagen. The response of lipocytes to type I collagen could then perpetuate the activated state. Alternatively, the primary event might be disruption of the normal subendothelial matrix. Matrix proteins secreted by inflammatory cells (50), Kupffer cells (51), or lipocytes (52) are capable of degrading basement membrane, potentially generating a matrix that activates lipocytes in the manner of cells cultured on plastic or type I collagen.

The effects of EHS on lipocyte function are due to its specific composition and/or structure and not solely to its mechanical properties as a gel because cells on a type I collagen gel are phenotypically similar to those on uncoated plastic. We have shown here and previously (16) that lipocytes on laminin, type IV collagen, heparan sulfate proteoglycan, and fibronectin substrata also are morphologically similar to cells on plastic, indicating that for maintaining a quiescent state the cells require a complete basement membrane-like substratum. Support of liver-specific function in hepatic parenchymal cells requires a complete basement membrane-like substratum. Support of liver-specific function in hepatic parenchymal cells requires a complete basement membrane-like substratum. Support of liver-specific function in hepatic parenchymal cells requires a complete basement membrane-like substratum.

The functional effects of substrata are paralleled in our study by changes in cell shape, as has been noted in studies of other connective tissue cells (12, 13). A compact spherical configuration of lipocytes is associated with decreased fibrogenic and proliferative activity. Similarly, Gospodarowicz et al. (53) have shown that fibroblasts maintained as spheres (in suspension) fail to divide in response to serum factors (49). Benya and Shaffer (54) have demonstrated that re-expression of a differentiated phenotype by cultured chondrocytes occurs as the cells acquire a spherical morphology.

The cellular sources of collagen in normal liver are matters of current debate. If cells cultured on EHS gel most closely mimic the in vivo phenotype, the data for lipocytes and hepatocytes suggest that the two cell types in normal liver may produce similar amounts of collagen, although some of the collagen measured in hepatocyte cultures may derive from contaminating lipocytes (32). Regardless, collagen synthesis by activated lipocytes (on plastic) far exceeds that of hepatocytes, suggesting that in acute liver injury these mesenchymal cells are the principal source of type I collagen, which is predominant in pathologic fibrosis.

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REFERENCES

Regulation of Lipocyte Matrix Phenotype