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Cooperation of Ito Cells and Hepatocytes in the Deposition of an Extracellular Matrix *In Vitro*

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Cellular and molecular mechanisms involved in the deposition of extracellular matrix components in both normal and fibrotic liver are still poorly understood. We have investigated the influence of cooperation between Ito cells and hepatocytes in matrix deposition* in vitro. *Immunoprecipitation of radiolabeled proteins from media of 5-day-old Ito cell primary cultures showed that these cells secreted high levels of the major basement membrane components, ie, collagen IV, laminin, and entactin/nidogen. By immunocytochemistry, precursors of basement membrane components were found intracellularly, but only scarce deposits were seen around the cells. When hepatocytes were added to 2-day-old Ito cell primary cultures, they established close contacts with Ito cells in less than 24 hours and expressed ZO-1, a tight junction-associated protein not detectable in standard hepatocyte culture. Cytochemistry analysis revealed an abundant extracellular matrix deposited over hepatocyte cords and between hepatocytes and Ito cells. Immunocytochemistry studies showed that this matrix contained laminin, fibronectin, and collagens proIII and IV. These data indicate that a high level of matrix protein synthesis by liver cells* in vitro *is not sufficient to induce extracellular matrix deposition, and that cell-cell interactions are strongly involved in this process. Hepatocyte/Ito cell co-culture, which may reflect the actual situation* in vivo, *represents a useful tool for studying liver fibrogenesis. (Am J Pathol 1993, 143:538–544)

In normal adult rodent and human livers, only small amounts of extracellular matrix are detectable in the sinusoids, and unlike most epithelial cells hepatocytes lack a continuous basement membrane.[1] This unique pattern of extracellular matrix deposition in the Disse's space contrasts with the abundant intracellular localization of matrix precursors and corresponding mRNAs in Ito cells, the main producers of matrix components.[2–4] However, conspicuous accumulation of extracellular matrix between hepatocytes and Ito cells occurs in the course of fibrosis and during the capillarization process that is associated with various chronic liver diseases.[3,5]

Active synthesis without concomitant pericellular accumulation of extracellular matrix components is also observed in Ito cell primary culture. Although these cells express increased amounts of both interstitial and basement membrane components after a few days in culture,[6–9] careful examination of data from immunocytochemistry studies[6,7] and immunoprecipitation of radiolabeled proteins[10,11] indicates that neosynthesized matrix proteins are only rarely associated with cell layers, and remain soluble in cell culture media. Similar observations have been made with hepatocytes; although they do not produce significant amounts of matrix proteins in normal adult liver, except for fibronectin,[12] they start producing soluble basement membrane components when placed in primary culture.[2,13] Taken together, these observations suggest that synthesis of matrix proteins and deposition in the intercellular spaces might be independent and under uncoordinated mechanisms. Since Ito cells are closely associated with hepatocytes *in vivo* and exhibit dramatic phenotype changes *in vitro* similar to those observed in fibrotic livers,[9] we hypothesized that a cooperation between Ito cells and hepatocytes could be involved in deposition of an insoluble matrix material. We show that, unlike standard pure cultures, co-cultures of Ito cells

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and hepatocytes deposit an abundant extracellular matrix, containing collagens, fibronectin, and laminin/entactin.

Materials and Methods

Antibodies

Anti-collagen IV, anti-laminin and anti-entactin antibodies were obtained after injection of purified antigens from the murine Engelbreth-Holm-Swarm (EHS) sarcoma into New-Zealand rabbits. Anti-fibronectin and anti-procollagen III antibodies were a generous gift from Dr. J.A. Grimaud (Institut Pasteur, Lyon, France). Anti-desmin antibodies were from Dako (Versailles, France). Anti-rat monoclonal antibodies against ZO-1, a tight junction-associated protein identified in the liver,[14] was obtained from Developmental Studies Hybridoma Bank (Baltimore, MD).

Cell Isolation and Culture

Ito cells were isolated from 6-month- to 1-year-old Sprague-Dawley male rats. Ito cells were purified after pronase and collagenase dissociation of the liver using a single-step density gradient centrifugation with Nycodenz (Nyegaard and Co., Oslo, Norway) at a final concentration of 11.4%.[6] Cells were plated in Dulbecco's minimum essential medium containing 10% fetal calf serum (FCS), penicillin (10 IU/ml) and streptomycin (50 µg/ml). The culture medium was renewed every day. Purity of Ito cell cultures was assessed on the basis of typical phase-contrast microscopic appearance, vitamin A specific autofluorescence,[6] red oil staining,[15] and positivity for desmin.[16] The lack of endogenous peroxidase activity and factor VIII positivity showed absence of contaminating Kupffer cells and sinusoidal endothelial cells, respectively.

Normal adult rat hepatocytes were obtained using the two-step collagenase perfusion method.[17] Co-cultures were set up by addition of freshly isolated hepatocytes to 2-day-old Ito cell primary cultures in a medium containing 75% minimum essential medium and 25% medium 199 (Gibco, Gaithersburg, Maryland), 10 µg/ml bovine insulin, 0.2% bovine serum albumin and 10% FCS. After 4 hours fresh medium containing 10^{-6} M hydrocortisone hemisuccinate was added. Pure Ito cell and hepatocyte cultures maintained in the same medium were used as controls.

Cytochemistry

Cell cultures were fixed with 4% paraformaldehyde solution in 0.1 M sodium cacodylate, pH 7.4, for 15 minutes at 4°C. Reticulin staining was performed as described by Gordon and Sweet.[18] Extracellular matrix components were localized using an indirect immunoperoxidase technique after 0.1% saponin treatment.[2] For ZO-1 immunolocalization, cells were washed with cold phosphate-buffered saline, gently scraped using a rubber policeman, embedded in Tissue-Tek ornithine carbamoytransferase solution (Miles Inc., Elkhart, IN), and frozen by immersion in liquid nitrogen. Cryostat sections (3–4 µm) were mounted on gelatinized slides. After saturation in phosphate-buffered solution containing 1% bovine serum albumin, sections were incubated with anti-ZO-1 antibodies for 1 hour and subsequently with tetraethyl rhodamine isothiocyanate (TRITC) conjugated goat anti-rat IgG at 4°C overnight. Control experiments were carried out using secondary antibodies only.

Immunoprecipitation

On day 5 of culture, Ito cells were incubated in a methionine-free medium containing 10% FSC and added with 40 µCi/ml ^{35}S-methionine (specific activity 1475 Ci/mmole, Amersham, Buckinghamshire, UK) for 12 hours. Specific antibodies were then added for 4 hours before addition of protein A-Sepharose, which had been previously soaked with nonlabeled medium for 1 hour. After extensive washes, beads were mixed with sample buffer containing 10% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol which was further loaded into a 5% SDS-polyacrylamide gel. Controls were obtained using culture medium incubated with nonimmune serum. For entactin immunoprecipitation, 0.1% SDS was added to the cell culture medium before incubation with anti-entactin antibodies.[19]

Results

Synthesis and Deposition of Extracellular Matrix Proteins in Short-Term Ito Cell Primary Cultures

Synthesis of collagen IV, laminin and entactin was investigated in 5-day-old Ito cell primary cultures by immunoprecipitation of ^{35}S-labeled proteins. Under reducing conditions, media of cultured Ito cells contained a major M_r 180,000 polypeptide reacting with

anti-collagen IV antibodies (Figure 1A). Bands of M_r 200,000 and 220,000 comigrating with the B1 and B2 chains of laminin and a M_r 380,000 polypeptide were resolved using anti-laminin antibodies (Figure 1B). In addition, a M_r 150,000 band was detected which was further identified as entactin by immunoprecipitation of radiolabeled medium with specific anti-entactin antibodies in the presence of 0.1% SDS (Figure 1C).

Immunolocalization of extracellular matrix components was performed in 5-day-old Ito cell primary cultures by indirect immunoperoxidase. Intracellularly, Ito cells were strongly labeled for laminin, collagen proIII and IV, and fibronectin. Extracellularly, no collagen IV (Figure 1D) and pro III (data not shown) stained material was detectable. A few deposits of laminin/entactin complexes were sometimes found (Figure 1E). Only fibronectin was found surrounding the cells (data not shown).

Deposition of extracellular matrix components in Ito cell-hepatocyte co-cultures

The role of cell-cell cooperation in matrix deposition was studied by co-culturing hepatocytes with Ito cells. When hepatocytes were added to 2-day-old Ito cell cultures, they rapidly attached, spread, and formed cords. Close contacts between both cell types were observed after 24 hours (Figure 2A). Hepatocytes exhibited typical refringent structures corresponding to newly formed bile canaliculi. Numerous lipid droplets were visible in the cytoplasm of Ito cells. Progressive phenotype changes occurred during the following week, including loss of lipid droplets and proliferation of Ito cells, and disappearance of refringent structures between hepatocytes (data not shown).

By immunofluorescence, ZO-1, a marker of tight junctions, was localized in hepatocytes after two days of co-culture (Figure 2B). No labeling was found in hepatocytes and Ito cells maintained in two-day-old pure cultures as well as in control immunocytochemistry experiments (data not shown).

Extracellular matrix distribution was analyzed using both the silver impregnation method and immunocytochemistry. Reticulin staining revealed abundant extracellular matrix in 4-day-old cocultures, but not in pure hepatocyte and Ito cell cultures at the same time (Figure 3). Deposition of extracellular matrix was preferentially located over hepatocyte cords and in between Ito cells and hepatocytes. No significant matrix deposition was found in areas where either hepatocytes or Ito cells did not form contacts. Characterization of extracellular matrix components by immunocytochemistry revealed that deposited matrix in co-culture contained laminin/entactin and collagen pro III and IV stained materials (Figure 4). Intracellularly, both cell types were positive for all these components. In control pure hepatocyte cultures, collagen pro III was not detectable and only little laminin and collagen IV was found (Figure 4). In control pure Ito cell primary cultures there was no significant deposition of these extracellular matrix components (Figure 1). Fibronectin was constantly present regardless of culture conditions (Figure 4).

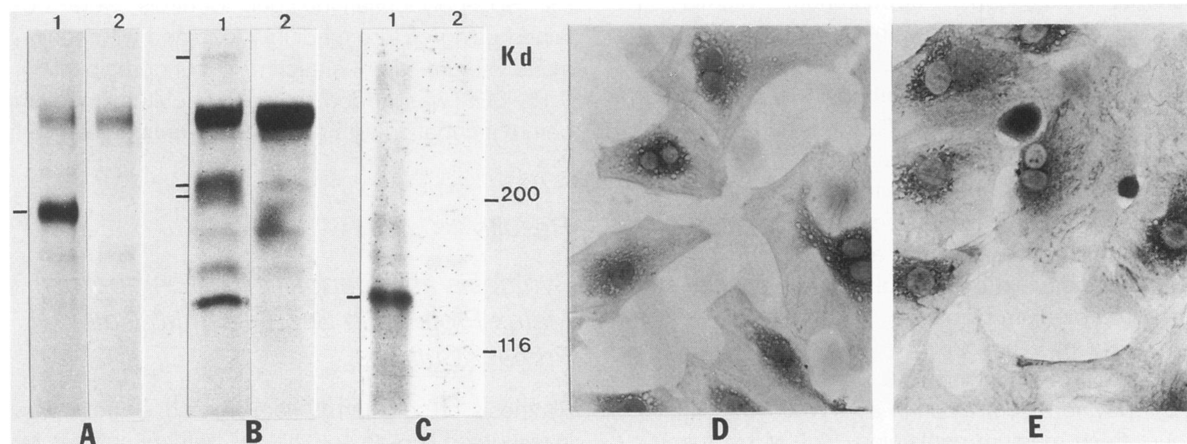

Figure 1. *Synthesis and deposition of extracellular matrix components in 5-day-old Ito cell primary cultures. Synthesis of basement membrane proteins was studied by immunoprecipitation of the ^{35}S-radiolabeled proteins from culture media using (lanes 1) anti-collagen IV* **(A)**, *anti-laminin* **(B)** *and anti-entactin* **(C)** *antibodies (lane 2 : non immune serum). Molecular weight standards are indicated on the right. The deposition of basement membrane components was studied by indirect immunoperoxidase of collagen IV* **(D)**, *and laminin/entactin complex* **(E)**. *(×960).*

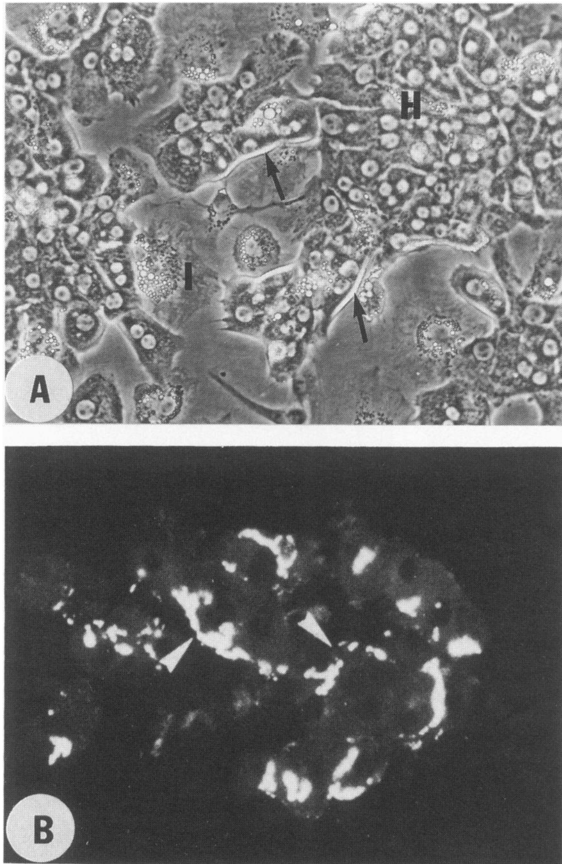

Figure 2. *Co-culture of Ito cells and hepatocytes. Phase-contrast microscopy* **(A)** *and immunolocalization of ZO-1* **(B)**. *In a 2-day old co-culture, Ito cells* **(I)** *and hepatocytes* **(H)** *form close contacts (arrows) and ZO-1 is localized in hepatocytes, only (arrowheads).* **(A)**: $\times 390$; **(B)**: ($\times 600$).

Discussion

Primary hepatocyte and Ito cell cultures are probably the most widely used model systems for studying normal and pathological hepatic functions. However, it is now clearly established that these cells undergo dramatic phenotype changes after a few days in culture. Thus, hepatocytes lose their adult specific functions, exhibit a fetal like state and then die;[20,21] Ito cells lose lipid droplets, become myofibroblastic and proliferate.[10] During the same period of time, both cells express higher amounts of extracellular matrix components than their normal counterparts *in vivo*. Hepatocytes which produce little matrix proteins in normal adult liver, if any, start producing basement membrane components after their isolation.[3,13] On the other hand, Ito cells express increased amounts of both interstitial and basement membrane components during their evolution towards a myofibroblastic state, i.e. after 4 to 5 days in culture.[6,7,9] However, when either hepatocytes[3] or Ito cells (the present study) are cultured under standard conditions, extracellular matrix proteins are only rarely associated with the cell layer, remaining mostly soluble in the culture medium. Here we show that, in contrast, co-culturing hepatocytes and Ito cells, which are closely associated *in vivo* and produce extracellular matrix components during fibrosis, results in a significant deposition of a complex biomatrix.

Deposition of extracellular matrix components occurs preferentially in areas where hepatocytes are associated with Ito cells, and not in areas where Ito cells are lacking, thus suggesting a role of intimate contacts between both cell types for matrix deposition. It is likely that cell-cell contacts are responsible for phenotypic modulations of hepatocytes. Indeed, the expression of ZO-1, a specific protein of tight junctions, was demonstrated in co-cultured hepatocytes only. Accordingly, the role of heterotypic cell-cell contacts in maintenance of liver-specific functions *in vitro* has been demonstrated in co-cultures of hepatocytes and rat liver epithelial cells from biliary origin.[22] Interestingly, in this co-culture model system maintenance of specific functions parallels the deposition of an abundant extracellular matrix. From co-culture experiments, one may hypothesize that the functional state of hepatocytes could influence extracellular matrix deposition in both normal and fibrotic livers. Thus, matrix deposition could result from a cooperation between cells which produce large amounts of matrix components, ie, Ito cells, and hepatocytes which regulate their deposition. Such a cooperation between epithelial and mesenchymal cells in extracellular matrix deposition in the developing intestine have been hypothesized from embryonic gut epithelial cells and mesenchyme-derived cell cocultures.[23]

The mechanisms involved in hepatic extracellular matrix deposition remain to be elucidated. It cannot be ruled out that in co-culture matrix protein synthesis is increased. However, both cell types in pure culture produce high levels of extracellular matrix components which are not significantly deposited, thus suggesting that synthesis and deposition of matrix proteins are independent mechanisms. Matrix deposition might involve key domains of plasma membrane. Recent findings indicate that expression of extracellular matrix receptors on hepatocyte surfaces depends on their functional state.[24] Accordingly, the distribution of integrins varies in fibrotic human livers.[25] While only $\alpha 1$ and $\alpha 5$ integrin chains were evidenced on hepatocytes in normal liver, $\alpha 2$, $\alpha 3$ and $\alpha 6$ chains were detected on hepatocytes in inflammatory and/or cholestatic livers. Other mechanisms resulting in assembly of extra-

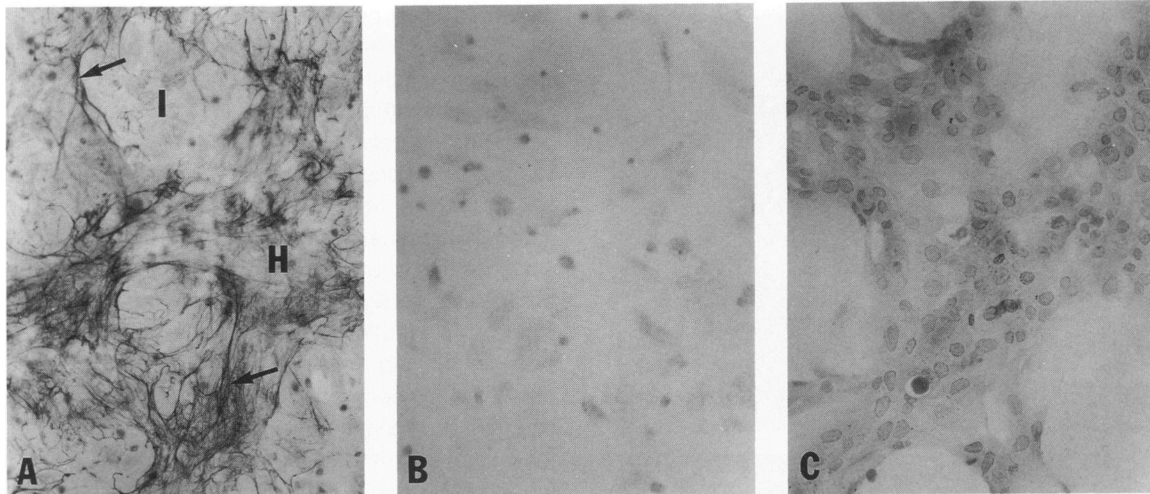

Figure 3. *Reticulin staining. In a 4-day-old co-culture* (A), *reticulin fibers (arrows) are localized on hepatocyte cords and between Ito cells* (I) *and hepatocytes* (H); *no staining is visible in both Ito cell* (B) *and hepatocyte* (C) *pure cultures.* (×460).

cellular matrix components between cells include synthesis of structurally intact molecules, intra and inter cross-linking of polypeptides, and self-aggregation of matrix proteins.[26] In standard pure culture conditions, hepatocytes and Ito cells may not coordinately express the whole set of matrix proteins and/or may synthesize incomplete molecules. For example, only laminin B chain genes are expressed in early hepatocyte and Ito cell pure cultures.[9,12] Since laminin polymerizes within basement membrane through interactions at the ends of its short and long arms,[27] coordinated synthesis of

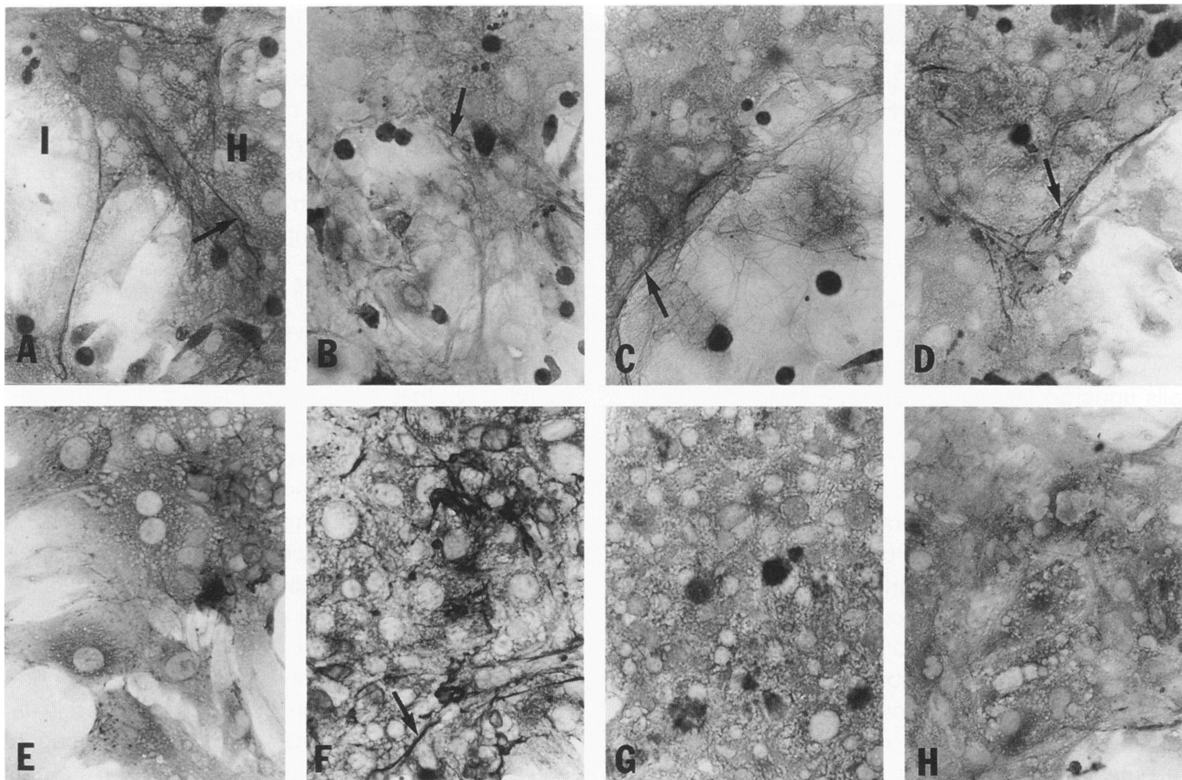

Figure 4. *Immunolocalization of extracellular matrix components. Laminin* (A,E), *fibronectin* (B,F) *and collagens proIII* (C,G), *and IV* (D,H) *were localized by indirect immunoperoxidase in both 4-day-old Ito cell/hepatocyte cocultures* (A,B,C,D) *and hepatocyte pure cultures* (E,F,G,H). *Laminin and collagens proIII and IV were deposited in co-cultures only (arrows). Fibronectin was detectable in both co-cultures and hepatocyte pure cultures* (B,F). (×720).

the three chains of laminin, or isoforms, might be achieved in co-culture only. Other key elements for assembly of basement membranes are both entactin/nidogen and perlecan which form links between laminin and collagen IV[28,29] and are produced *in vitro* by Ito cells (the present study) and hepatocytes,[30] respectively. Finally, soluble factors, eg, cytokines such as transforming growth factor β (TGFβ) may also be involved in extracellular matrix deposition. However, that deposition of extracellular matrix components occurs only in areas in which both cell types establish close contacts suggests a predominant role of cell-cell interactions in matrix deposition.

In conclusion, our study shows for the first time that cooperation(s) between Ito cells and hepatocytes is involved in the deposition of hepatic extracellular matrix. Hepatocyte / Ito cell co-cultures could represent a unique tool for studying the cellular and molecular mechanisms involved in extracellular matrix deposition in both normal and fibrotic livers.

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