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Differential expression of laminin chains in hepatic lipocytes

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The lipocyte is an important source of laminin in the normal liver. We have investigated the expression of the 3 chains of laminin in isolated rat lipocytes. Both B_1 and B_2 chains, but not A, were found in medium from 5-day-old lipocyte primary cultures by immunoblotting and immunoprecipitation of ^{35}S-labeled proteins after reducing SDS-polyacrylamide gel electrophoresis. An additional polypeptide of M_r=380 000 was identified by immunoprecipitation. Under non-reducing conditions only one M_r=900 000 band was revealed. High levels of B_1 and B_2 mRNAs were also demonstrated in 5-day-old cultured lipocytes while at the time of seeding, only B_2 chain mRNAs were clearly detectable. A chain mRNA was constantly absent. These results suggest that lipocytes produce a variant form of laminin in primary culture and that the M_r=380 000 polypeptide could be unrelated to the A chain of laminin.

Laminin; Lipocyte; Liver; Cell culture; mRNA

1. INTRODUCTION

Laminin is the major noncollagenous glycoprotein of all basement membranes [1,2]. The most well studied laminin is from the mouse Engelbreth-Holm-Swarm (EHS) tumor [1] which was found to be composed of three genetically distinct chains, A (M_r=400 000), B_1 (M_r=210 000) and B_2 (M_r=200 000), which are held together by disulfide bonds in a cross-shaped molecule [2]. When used as a substrate in vitro, EHS laminin may exert various biological activities including promoting adhesion, migration, growth, and differentiation of a variety of cells [3–5]. Laminin of non-neoplastic origin may exhibit structural differences, including changes in the A, B_1 and B_2 chain ratio and/or the presence of isoforms. Thus, many cells in culture and various tissues contain only small amounts of A chain, if any [6–9]. In addition, variants of laminin that contain polypeptides different from those found in the murine EHS molecule have been identified in several tissues, including placenta, heart and neuromuscular junctions [10–15].

In the liver, laminin is present mainly around vessels and bile ducts. It is also sparsely deposited in the space of Disse, at the contact sites of hepatocytes [16]. In vitro, laminin is a potent regulator of the behaviour and function of hepatocytes [17]. However, the relevance of these findings may depend on the actual structure of the molecule in vivo. Indeed, hepatocytes may interact differently with the 3 chains of laminin [18], thereby resulting in the induction of specific regulatory signals. Since lipocytes are both closely located to hepatocytes and one of the major sources of laminin in the liver [16,17], we have studied the expression of the 3 chains of these cells after their isolation. We show that hepatic lipocytes in culture express B_1 and B_2 chains and a polypeptide of M_r=380 000 which probably differs from the A chain of the EHS laminin.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Cell culture medium was from Gibco. Collagenase and DNAse were purchased from Boehringer and pronase E from Merck. Nycodenz was obtained from Nyegaard and Co. (Oslo, Norway). Anti-laminin antibodies were obtained following injection of laminin purified from the EHS tumor in a New Zealand rabbit (Levavasseur et al., unpublished). Anti-desmin antibodies were from Dako. L-[^{35}S]methionine (specific activity 1475 Ci/mmol) was obtained from Amersham.

2.2. *cDNA probes*

Laminin A chain cDNA probe was a 1.2 kb *Eco*RI fragment of 1A-E3 cloned from an EHS tumor cell cDNA library [19]. Laminin B_1 chain cDNA probe was a 0.5 kb *Eco*RI–*Hin*dIII fragment of P24 cloned from mouse differentiated F9 cell cDNA library [20]. Laminin B_2 chain cDNA probe was a 1.5 kb *Eco*RI–*Hin*dIII fragment of P7 cloned from an F9 cell cDNA library [21]. The parietal yolk cell line (PYS, a gift from Dr P. Burbelo, NIDR, Bethesda) was used as a positive control for Northern blot hybridization with the laminin A chain cDNA probe.

2.3. *Cell culture*

Ito cells were obtained from 6-month- to 1-year-old Sprague–Dawley male rats. Non parenchymal liver cells were first isolated using the pronase–collagenase method [22,23] with slight modifications. Ito cells were purified using a single-step density gradient centrifugation with Nycodenz at final concentration of 11.4%. Cells were plated in Dulbecco minimal essential medium containing 10% fetal calf serum (FCS). Culture medium was renewed daily. Purity of the Ito cell

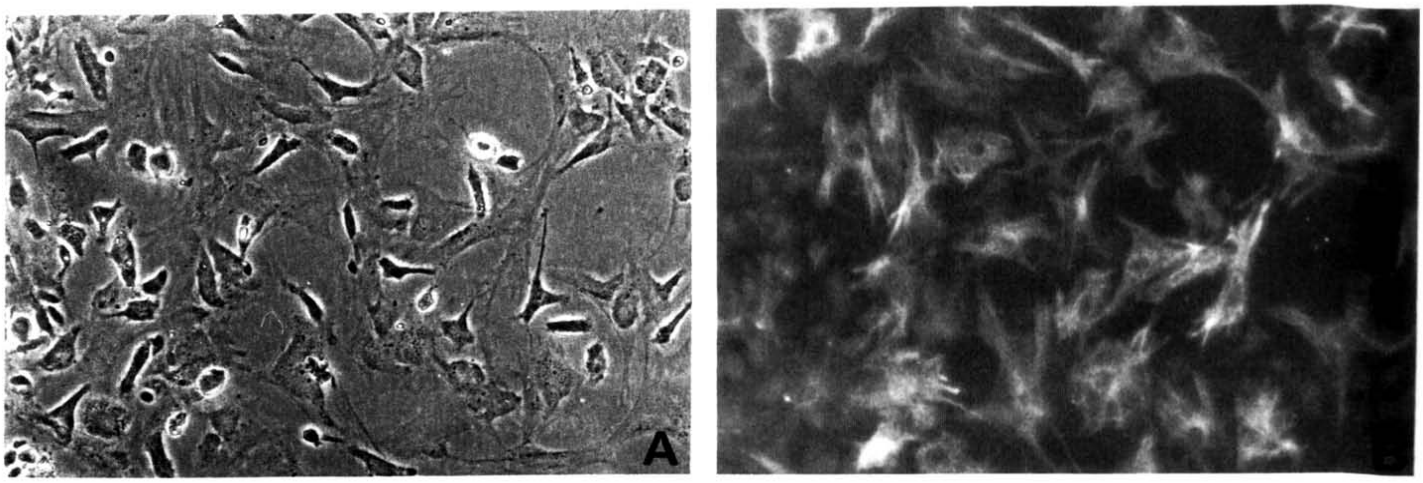

Fig. 1. (A) Phase contrast microscopic observation of 5-day-old hepatic lipocytes culture. (B) Immunofluorescent staining of desmin in the same field (× 240).

cultures was assessed on the basis of typical phase-contrast microscopic appearance, vitamin A-specific autofluorescence [23,24], red oil staining [25] positivity for desmin [23,26] and lack of endogenous peroxidase activity and factor VIII. All experiments were performed 5 days after cell seeding, except for Northern blot analyses which were performed with both freshly isolated cells and 5-day cultured lipocytes.

2.4. *Fixation and immunocytochemistry*

Cell cultures were rapidly washed with phosphate buffered saline (PBS) pH 7.5, and then fixed with a 4% paraformaldehyde solution buffered with 0.1 M cacodylate, pH 7.4, for 45 min at 4°C. Subsequently, desmin and factor VIII were localized using indirect immunofluorescence and immunoperoxidase techniques, respectively.

2.5. *Immunoprecipitation*

Cell cultures were incubated with 40 μCi [^{35}S]menthionine per ml of methionine-free medium containing 10% FCS. After 12 h, the medium was removed and supplemented with 1 mM phenylmethylsulfonyl. The medium was centrifuged and then incubated for 4 h with anti-laminin antibodies prior to addition of protein A-Sepharose, which has been first soaked with non-labeled medium for 1 h. Then, beads were washed twice with PBS, followed by Ripa buffer containing 10 mM Tris-HCl, pH 7.2, 0.13 M NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.01% sodium dodecyl sulfate (SDS) and 10 mM Tris-HCl, pH 7.4. Subsequently, the beads were mixed with sample buffer containing 10% SDS with or without 5% β-mercaptoethanol, and then loaded on a 5% SDS-polyacrylamide gel. Culture media incubated with non-immune serum were used as controls.

2.6. *Immunoblotting*

Proteins were precipitated from the medium with 10% trichloroacetic acid. Pellets were washed twice with acetone, dried and then mixed with sample buffer containing or not 5% β-mercaptoethanol. Proteins were resolved on a 5% SDS-polyacrylamide gel and transferred onto nitrocellulose sheets. Then filters were incubated for 2 h with Tris-buffered saline (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl) containing 1.5% BSA and 1.5% dried milk. Then the sheets were incubated for 1 h with anti-laminin antibodies diluted (1/250) in the same buffer containing 0.25 M NaCl and 0.3% Nonidet. Filters were washed twice and then incubated with horseradish peroxidase-labeled anti-rabbit antibodies. Revelation was performed using a 5% chloronaphtol solution. Laminin purified from EHS sarcoma was used as standard.

2.7. *mRNA analysis*

Total RNAs were isolated using the guanidinium-thiocyanate/cesium chloride method [27]. RNAs (10 μg per lane) were resolved by electrophoresis on a 1% agarose slab gel with 10 mM sodium phosphate pH 7.4 containing 1.1 M formaldehyde and transferred onto nitrocellulose sheets. Filters were hybridized in 3×SSC, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% SDS, 0.1% BSA and 10% dextran sulfate. The filters were washed for 30 min with 3×SSC at 65°C, and then with 1×SSC, 0.1% SDS for 30 min at 65°C.

3. RESULTS

Laminin production was studied in 5-day-old cultured hepatic lipocytes. At that time the cells were well spread and they still exhibited typical morphological appearance with refringent globules and spindle-like extensions under phase-contrast microscopy (Fig. 1A). Over 95% of the cells were positive for both desmin (Fig. 1B) and vitamin A autofluorescence (data not shown). Endogenous peroxidase activity was undetectable while no cell was found to stain for factor VIII antibodies (data not shown).

Specificity of laminin antibodies was assessed by immunoblotting against the purified basement membrane components laminin and collagen IV. Only 2 bands

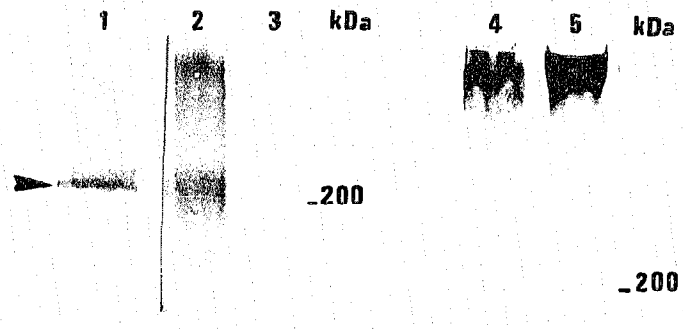

Fig. 2. Immunoblotting of laminin. Media from 5-day-old hepatic lipocyte cultures (lanes 1 and 4), and laminin (lanes 2 and 5) collagen IV (lane 3) extracted from the EHS tumor were resolved by 5% SDS-polyacrylamide gel electrophoresis with (lanes 1, 2 and 3) or without (lanes 4 and 5) 5% β-mercaptoethanol. Arrow, laminin B chains. Standard molecular weights, × 10^{-3}.

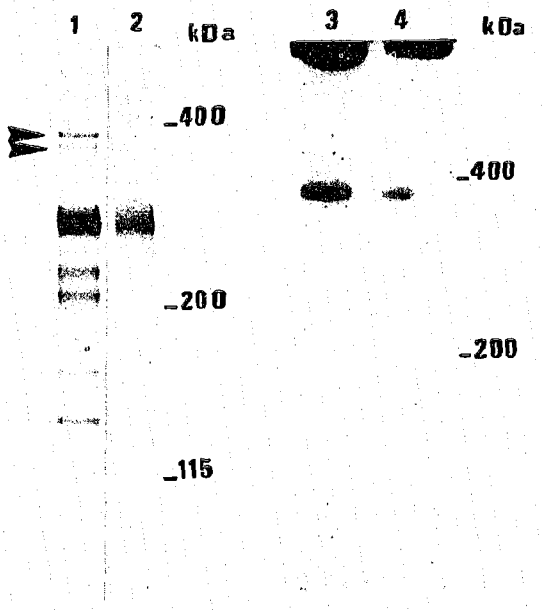

Fig. 3. Immunoprecipitation of ^{35}S-labelled laminin resolved by 5% SDS-polyacrylamide gel electrophoresis with (lanes 1 and 2) or without (lanes 3 and 4) 5% β-mercaptoethanol. Lanes 1 and 3, media from 5-day-old hepatic lipocyte cultures; lanes 2 and 4, non-immune serum. Double arrow, M_r 380 000 polypeptide. Standard molecular weights, $\times 10^{-3}$.

whose sizes correspond to the A chain (upper band) and the B_1 and B_2 chains (lower band) were visualised with purified laminin while anti-laminin antibodies did not react with collagen IV (Fig. 2, lanes 2 and 3).

The presence of laminin in media from 5-day-old cultures was assessed by immunoblotting. After reducing SDS-electrophoresis and transfer to nitrocellulose filters, incubation of sheets with anti-laminin antibodies revealed the presence of a band co-migrating with the B chains of EHS laminin (Fig. 2, lane 1). Under non-reducing conditions, only one M_r=900 000 band co-migrating with non-reduced purified laminin was evident (Fig. 2, lane 4).

Laminin synthesis was studied in these cultures by immunoprecipitation of the ^{35}S-labeled proteins (Fig. 3). Under reducing conditions, media of cultured lipocytes contained 2 radiolabeled polypeptides of M_r=200 000 and 220 000 respectively, comigrating with

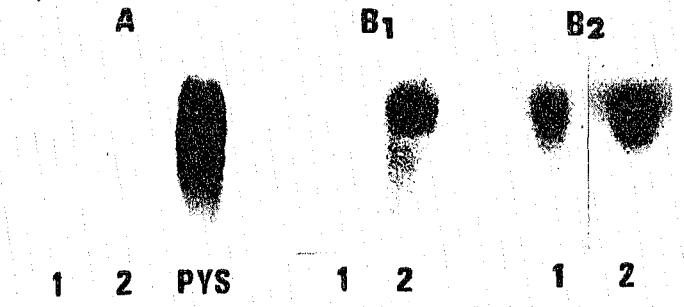

Fig. 4. Northern blot analysis of laminin A (A), B_1 (B_1) and B_2 chain (B_2) mRNA in freshly isolated lipocytes (lane 1) and 5-day-old cultured hepatic lipocytes (lane 2). The filter, hybridized with the A cDNA probe, was exposed for 10 days. PYS, parietal yolk sac rat tumor.

the B chains of purified laminin. In addition, an M_r=380 000 band had migration distance close to that of the A chain of EHS laminin. This polypeptide was often resolved as a doublet (Fig. 3, lane 1). An additional radiolabeled band which probably corresponds to entactin had an estimated size of M_r=160 000. Under non reducing conditions, only one M_r=900 000 radiolabeled band comigrating with the EHS laminin was revealed from the immunoprecipitated ^{35}S-medium (Fig, 3, lane 3).

The steady-state levels of laminin A, B_1 and B_2 chain mRNAs from both freshly isolated and 5-day-old cultured hepatic lipocytes were studied by Northern blot hybridization. Only laminin B_2 chain mRNA was clearly detectable in freshly isolated lipocytes (Fig. 4). After 5 days, cultured lipocytes contained abundant laminin B_1 and B_2 chain mRNAs while A chain mRNA was constantly undetectable, even after a 10 day film exposure (Fig. 4).

4. DISCUSSION

Using both immunoblotting and immunoprecipitation of released radiolabeled proteins, we clearly show that lipocytes synthetize and secrete high levels of laminin B chains in culture. Their production was supported by the presence of B_1 and B_2 chain mRNAs. These results confirm and extend previous finding suggesting that lipocytes are one of the major cellular sources of laminin in the liver [16,28,29].

Our data suggest that cultured lipocytes express a variant form of laminin composed of B_1, B_2 and an M_r=380 000 polypeptide not related to the A chain. Indeed, neither A chain nor A chain mRNA were visualized by immunoblotting and Northern blotting, respectively. However, an M_r=380 000 polypeptide was found to be immunoprecipitated with anti-laminin antibodies. Since only an M_r=900 000 polypeptide was visualized under non-reducing conditions, the M_r=380 000 polypeptide probably formed a complex with B_1 and B_2 chains by disulfide bonds. Our results contradict the conclusions from Maher et al., that hepatic lipocytes synthetize an intact from of laminin in culture [28]. However, neither immunoblotting nor Northern blotting were performed in this study. Further works are needed to investigate whether the M_r=380 000 polypeptide corresponds to a polypeptide not related to the A chain of the EHS laminin, e.g. merosin, or a polypeptide generated by alternative splicing of the A chain transcript. It is noteworthy that homologues of laminin chains have been found in several tissues including placenta [14], neuromuscular junction [13], and heart [12]. In addition, cells in culture such as adipocytes [11] endothelial cells [15] and hepatoma cells [30] may contain an A-like molecule not related to the A chain of EHS laminin.

We have previously shown by immunoelectron mi-

croscopy that lipocytes are laminin producers in both normal and fibrotic livers [16]. Only B_2 chain mRNAs were clearly detectable in freshly isolated lipocytes by Northern blotting, thus suggesting that laminin genes are not coordinately expressed by these cells in vivo. It cannot be excluded, however, that these genes are transcribed at low level and/or mRNAs are rapidly processed and degraded during cell isolation. This observation does not support those from Milani et al. [31] showing the presence of B_1 chain gene transcripts in sinusoidal cells by in situ hybridization on liver slices. Since endothelial cells also produce laminin in the liver [16,29], and since it is difficult to distinguish the endothelial cells and lipocytes under light microscopy, further study using purified endothelial cells from liver is required to clarify this point.

Lower amounts of B_2 chain mRNA were found in freshly isolated cells when compared with 5-day-old lipocyte cultures, while B_1 chain mRNA was clearly detectable in culture only. It has been shown that hepatic lipocytes undergo changes in morphology and gene expression when cultured in vitro. For example they express more collagens and proteoglycans after several days than in short-term primary culture [24,32]. Similar observations have been made with hepatocyte primary cultures. These cells, which do not synthesize significant amounts of collagens and laminin in the normal liver, start producing the B_1 and B_2 chains after a few hours in culture [33].

In summary, our data indicate that lipocytes express a variant from of laminin which lacks the A chain. Further work is required to characterize the M_r=380 000 chain that is synthesized by lipocytes in culture. These data should provide new insights into the actual form of laminin in the liver and, consequently, its effects on hepatocyte behavior.

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