

## Enterocytic differentiation of the human Caco-2 cell line is correlated with down-regulation of fibronectin and laminin

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### Abstract

Human intestinal Caco-2 cells were used to examine the expression of fibronectin (FN) and laminin (LN) during enterocytic differentiation. Combination of immunoprecipitation, Western and Northern blotting revealed that Caco-2 cells expressed a classical FN and a variant form of LN besides B1 and B2 chains, LN contained a 350-kDa heavy chain instead of the 400-kDa A chain. Throughout Caco-2 cell differentiation, FN and LN synthesis decreased at both mRNA and protein levels. These data indicate that enterocytic differentiation involves both transcriptional and/or post-transcriptional down-regulation of FN and LN gene expression.

**Key words:** Caco-2 cell, Enterocytic differentiation, Fibronectin, Laminin, Down-regulation

### 1. Introduction

The onset of epithelial cell differentiation requires close association of the cells with extracellular matrix components that constitute a basement membrane [1,2]. Substantial data suggest that the interactions of epithelial cells with basement membrane components play a crucial role in the regulation of cell morphology and growth, adhesion and differentiation [3], as well as transformation [4]. In the intestine, a continuous basement membrane separates the endodermally-derived intestinal epithelium from the mesenchymally-derived lamina propria [5]. On this basement membrane, the crypt base columnar cells, considered as the stem cells of the intestinal epithelium, undergo a constant proliferation and migration along the crypt-villus axis, and differentiate into an intestinal epithelium mainly composed of absorptive cells [6]. Once they reach the tip of the villi, the enterocytes become less adhesive to extracellular matrix proteins, and are released into the intestinal lumen. This process, which suggests modification of cell-to-cell and cell-to-matrix adhesion, involves changes in the biosynthesis of basement membrane components [7]. Among these components, FN and LN, two major non-collagenous glycoproteins playing a role in basement mem-

brane organization, may be critical for enterocytic differentiation.

Cellular FN was initially reported as a cell surface component of cultured fibroblasts [8]. Since then, it has been found on many different cell types including epithelial cells [9]. The FN molecule is a dimer, apparently a heterodimer of two similar but not identical 220-kDa subunits linked near the carboxyl termini by disulfide bonds [10]. LN, initially purified from the Engelbreth-Holm Swarm (EHS) tumor, is a multidomain protein (900 kDa), made up of three genetically distinct polypeptide chains: A (400 kDa), B1 (200 kDa) and B2 (220 kDa) linked by disulfide bonds, which form a unique cruciform shape when viewed by electron microscopy after rotary shadowing [11].

The human colonic cell line, Caco-2, shown to spontaneously express in culture an enterocyte-like phenotype [12] provides a very useful system in which to study the onset of enterocytic differentiation. This process has been well characterized on the basis of morphological and functional criteria [12–14] as well as changes in glycosaminoglycan biosynthesis [15,16]. However, to our knowledge, no study investigated whether Caco-2 cell differentiation is associated with changes in the expression of FN and LN. To address this issue, FN and LN were studied in non-differentiated and differentiated Caco-2 cells with respect to (1) steady-state mRNA levels, (2) polypeptide chain expression, and (3) synthesis

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and relative distribution between the cell and the extracellular medium

## 2. Materials and methods

### 2.1. *Caco-2* cell differentiation

Caco-2 cells were obtained from Dr. A. Zweibaum (INSERM U178, Villejuif, France) and cultured as reported [12]. On day 5 after plating, cells are in a non-differentiated state [12,13]. Enterocytic differentiation is a growth-related phenomenon starting as soon as confluency is reached (day 6). At day 12, Caco-2 cells are in a differentiated state characterized by (1) morphological differentiation (polarization of the cells, presence of apical brush borders and functional complexes) [12,13], (2) functional differentiation (formation of domes) [12,15], and (3) enzymatic differentiation (brush border hydrolase activities) [13,14]. Experiments were performed on days 5, 6 and 12 of culture.

### 2.2. *cDNA* probes, Northern blot hybridization

The LN B1 cDNA probe was a 500 bp *EcoRI*–*HindIII* fragment of P24 cloned from a mouse differentiated F9 cell cDNA library [17]. The LN B2 cDNA probe was a 1.5 kb *EcoRI*–*HindIII* fragment of P7 cloned from a F9 cell cDNA library [18]. The LN A chain cDNA probe was a 1.2 kb *EcoRI*–*SacI* fragment of 1A-E3 cloned from EHS tumor cell cDNA library [19]. EHS cells were used as a positive control for Northern blot hybridization with the LN A chain cDNA probe. Total RNA preparation, electrophoresis, transfer to Hybond N (Bio-Rad) and hybridization were as reported [20]. The membranes were exposed to X-Omat AR-5 Kodak films. RNA was quantified by densitometric scanning of FN, and LN B1 and LN B2 bands. Correction for loading was performed by dividing the densitometries of these bands by the densitometries of 28S rRNA bands in each lane obtained during subsequent hybridization with a 28S rRNA oligonucleotide probe labeled with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP.

### 2.3. [ $^{35}$ S]Methionine metabolic labeling and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

At each indicated time of culture, cells were incubated with 30  $\mu$ Ci/ml of [ $^{35}$ S]methionine (1,000–1,100 Ci/mmol, New England Nuclear, Boston, USA). After a 24-h labeling period, the radioactive medium was removed and supplemented with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide) and 20 mM EDTA. Cells were harvested and immediately lysed with RIPA buffer as described previously [21] in the presence of the protease inhibitors mentioned above. Media and cell lysate fractions were incubated with a 1:200 dilution of antibodies specific for either FN or LN (Telios, San Diego, USA) prior to addition of protein A-Sepharose [20] (Gibco, BRL). Non-immune serum served as control. In order to quantify FN and LN synthesis throughout Caco-2 cell differentiation, aliquots of immunoprecipitates corresponding to equal numbers of cells from each culture were loaded on the same gel and run under reducing conditions (100 mM dithiothreitol) [22], on 4–5% SDS-polyacrylamide gels with a 3% polyacrylamide stacking gel. After visualization of radiolabeled FN and LN polypeptides by autoradiography, the corresponding bands in the gels were cut out, dissolved in 30% hydrogen peroxide ( $H_2O_2$ ) overnight at 60°C and counted.

### 2.4. Western blotting

Cell lysates and media from unlabeled Caco-2 cells were analyzed by SDS-PAGE as described above. Western blotting was performed as previously described [20], using anti-FN or anti-LN (1:200) antibodies, anti-rabbit IgG coupled to peroxidase (Bioss, France) (1:4000) and 0.01%  $H_2O_2$  with 0.05% 4-chloro-1-naphthol.

## 3. Results

### 3.1. Steady-state levels of FN and LN mRNAs in differentiating Caco-2 cells

To determine whether Caco-2 cell differentiation is associated with changes in FN and LN expression, the

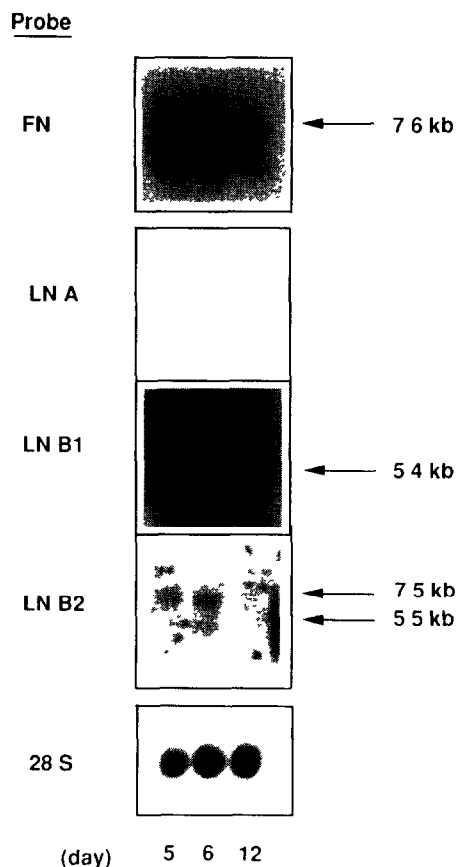


Fig. 1. Northern blot analyses. Total RNAs were prepared from Caco-2 cells on days 5, 6 and 12 of culture and analyzed (20  $\mu$ g/lane) by Northern blotting using cDNA probes for FN and LN A, LN B1 and LN B2. Filters were subsequently hybridized with a 28S rRNA probe.

steady-state levels of FN mRNAs and LN mRNAs corresponding to LN A, LN B1 and LN B2 chains were examined on days 5, 6 and 12 of culture. As shown in Fig. 1, a 7.6-kb FN transcript, a 5.4 kb LN B1 and two 7.5- and 5.5-kb LN B2 transcripts were detected in Caco-2 cells, regardless of the time of culture. By contrast, no transcript for LN A heavy chain was detected in Caco-2 cells, at any time considered, even after a 10 days film exposure. As determined by densitometric scanning of the Northern blots and normalization of the results with respect to the 28S signal, a marked decrease in the steady-state levels of FN mRNAs as well as of LN B1 and LN B2 mRNAs was observed in differentiated cells vs. non-differentiated cells. Thus, at day 12, i.e. in differentiated Caco-2 cells, the levels of FN, LN B1 and LN B2 mRNAs were only 12, 19 and 22% of the respective levels determined at day 5.

### 3.2. Characterization of FN and LN polypeptides synthesized and secreted in differentiating Caco-2 cells

$^{35}$ S-labeled FN and LN polypeptides synthesized and secreted by Caco-2 cells throughout the differentiation process were immunoprecipitated with specific FN and

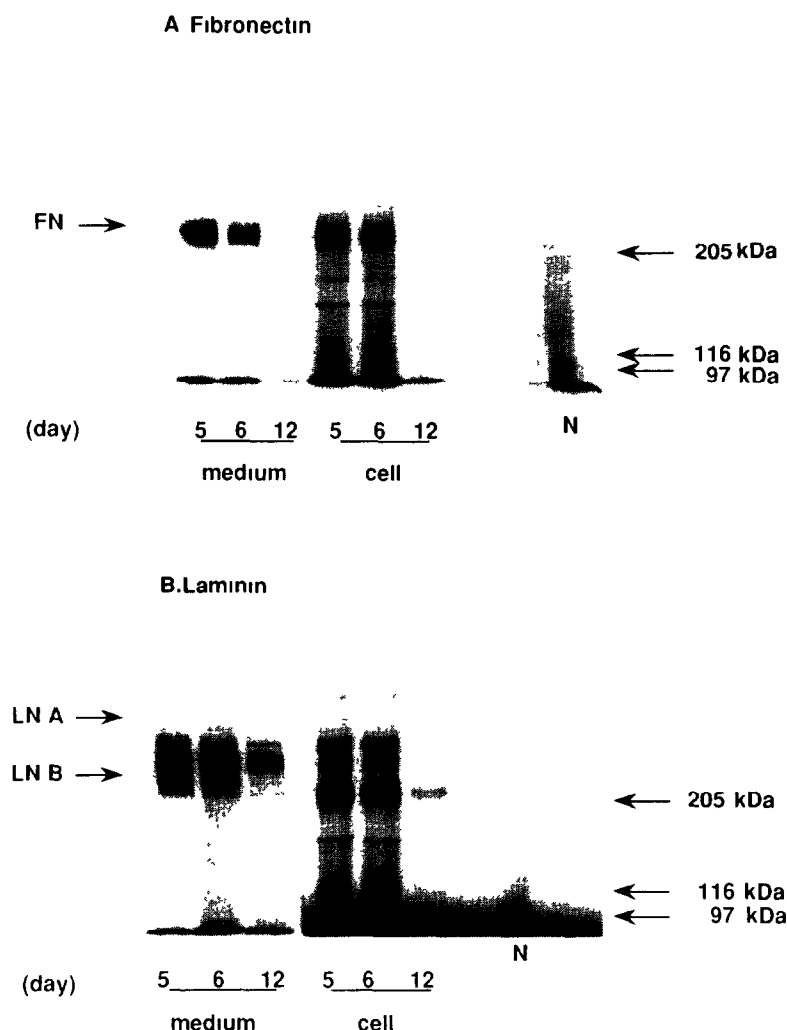


Fig. 2 Immunoprecipitation of  $^{35}\text{S}$ -labeled proteins isolated from either media or cell lysates. On days 5, 6 and 12, Caco-2 cells were labeled with  $^{35}\text{S}$ -methionine. Cell lysate (cell) and secreted (medium) fractions were immunoprecipitated with anti-FN antiserum (panel A), anti-LN anti-serum (panel B), or non-immune serum (N). The immunoprecipitates were fractionated on a 4.5% SDS-polyacrylamide gel and an autoradiogram of the dried gels was taken. Migration positions of either FN or of LN A and LN B subunits isolated from EHS tumor are indicated by arrows. Molecular weight markers: myosin (205 kDa),  $\beta$ -galactosidase (116 kDa) and phosphorylase b (97 kDa) are indicated.

**LN antibodies.** A 220-kDa FN polypeptide, co-migrating with a purified FN subunit, was resolved from both cell lysates and media at any time considered (Fig. 2A). Consistent with the results of Northern blot analysis, the labelling of the 220-kDa FN polypeptide was poorly detected on day 12, indicating a marked decrease in the synthesis and secretion of FN in differentiated Caco-2 cells.

Analysis of LN polypeptides from cell lysates and media revealed a closely spaced doublet at approximately 350 kDa, and intermediate band at 280 kDa and another doublet at 195–205 kDa co-migrating with the B-subunits of purified EHS LN (Fig. 2B). Differentiated Caco-2 cells showed markedly decreased synthesis of LN polypeptides. The unusual heavy chain of 350 kDa immunoprecipitated with anti-LN antibodies was further analyzed by Western blot (Fig. 3). In these experiments, only cell lysates, in which the LN polypeptides

were found to be more abundant, were examined. As shown in Fig. 3, the 350-kDa polypeptide immunoreacted with a polyclonal anti-LN antibody. The 280-kDa band which may be a protein bound to LN with high affinity as reported for other basement membrane components [23] was no longer detectable. So, the results of Western blot hybridization provide evidence for the presence in Caco-2 cells of a 350-kDa polypeptide immunologically related to LN, and confirm the absence of the traditional LN A chain, as revealed by Northern blot analysis.

### 3.3 Quantification of synthesis and secretion of FN and LN in differentiating Caco-2 cells

To quantify the amounts of FN and LN synthesized and secreted by differentiating Caco-2 cells, radioactivities of FN and LN polypeptides obtained at days 5, 6 and 12 were evaluated by excising and counting the corre-

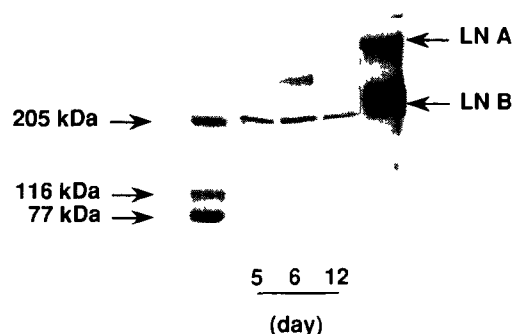


Fig 3 Western blot of cell lysate extracts with anti-LN antiserum. Cell lysate extracts from Caco-2 cells on days 5, 6 and 12 were fractionated by SDS-PAGE and electrophoretically transferred onto nitrocellulose. The antigen-antibody complexes were detected with a peroxidase-labeled anti-IgG. Left lane: molecular weight markers, myosin (205 kDa),  $\beta$ -galactosidase (116 kDa) and bovine serum albumin (77 kDa). Right lane: EHS LN.

sponding SDS polyacrylamide bands. Analysis of the quantitative data (Table 1) showed that both synthesis and secretion of FN and LN were markedly reduced in differentiated cells. In addition, the ratios of the amounts of FN and LN secreted relative to those of FN and LN synthesized were identical at any time considered.

#### 4. Discussion

The present study demonstrates that Caco-2 cell enterocytic differentiation is associated with dramatic decreases in FN and LN expression.

Caco-2 cells synthesized a 220-kDa FN polypeptide, similar to that expressed by a variety of cells in culture [8,9]. With respect to the LN molecules, Caco-2 cells expressed LN B1 and LN B2 chains but not the LN A chain, at both the mRNA and protein levels. The fact that LN A was undetectable in Caco-2 cells is not unusual, since the absence of LN A mRNA has been described in other cell lines [20,24,25]. Instead of the classi-

cal LN A chain, Caco-2 cells expressed a 350-kDa heavy chain, which was immunologically related to LN.

Several variants of LN with different chain composition and biological functions have been reported to date: the merosin M chain, that is homologous to, and takes place of the A chain in the LN molecule, is restricted to the human placenta, Schwann cells and striated muscles; the S-LN, a homologue of the B1 chain is selectively associated with the synaptic cleft of the neuromuscular junction; the K-LN, immunologically and structurally related to the 200-kDa subunit of kalinin is localized in the dermal-epidermal junction in skin and the novel LN B1 chain is produced by avian eye [23]; these variants have the B2 chain in common [26]. On the basis of sequence identity between S and B1 chains (50%), and between the A chain and the G domain of merosin (41%), it has been hypothesized that the S-chain and merosin are the result of recent gene duplication of the primordial gene generating LN A, LN B1 and LN B2 [26]. Our paper provides evidence for the presence in Caco-2 cells of a LN molecule which has not been described so far and which could represent a LN variant. Whether the 350-kDa polypeptide is a product of an altered version of the gene coding for the A or M subunit or the product of another gene remains to be elucidated.

The expression of FN and LN decreased simultaneously throughout Caco-2 cell differentiation, suggesting coordinated regulation of the genes encoding for these glycoproteins. A decrease in the expression of FN and LN was similarly observed during differentiation of other cell types [27,28]. Furthermore, changes in FN or LN synthesis have been reported in intestinal cell cultures [29] or during intestinal development [30]. Most importantly, *in vivo* studies into rat intestinal basement membrane synthesis have shown that LN B1 and LN B2 mRNAs were enriched in undifferentiated crypt cell fractions, whereas the steady-state level of these transcripts decreased in the superficial villus enterocyte cell fractions [7]. Together, these results point to decreases in FN mRNAs and LN B1 and LN B2 mRNAs associated with

Table 1  
Distribution of FN and LN in cell lysate and medium fractions from differentiating Caco-2 cells

	FN ( $^{35}$ S-radioactivity)			LN ( $^{35}$ S-radioactivity)		
	d 5	d 6	d 12	d 5	d 6	d 12
Cell lysate	6715 $\pm$ 390 (71.1 $\pm$ 3.1)	7015 $\pm$ 930 (68.5 $\pm$ 3.2)	490 $\pm$ 40 (62.0 $\pm$ 4.0)	15755 $\pm$ 1390 (81.2 $\pm$ 2.9)	17510 $\pm$ 1795 (80.0 $\pm$ 2.3)	5500 $\pm$ 575 (74.4 $\pm$ 2.8)
Medium	2735 $\pm$ 380 (28.9 $\pm$ 3.1)	3210 $\pm$ 395 (31.5 $\pm$ 3.2)	300 $\pm$ 27 (38.0 $\pm$ 4.0)	3615 $\pm$ 480 (18.8 $\pm$ 2.9)	4380 $\pm$ 510 (20.0 $\pm$ 2.3)	1890 $\pm$ 245 (25.6 $\pm$ 2.8)
Total	9450 $\pm$ 515 (100)	10220 $\pm$ 1160 (100)	760 $\pm$ 50 (100)	19300 $\pm$ 1445 (100)	25220 $\pm$ 4050 (100)	7390 $\pm$ 660 (100)

The amounts of  $^{35}$ S-labeled FN and of  $^{35}$ S-labeled LN in the designated fraction were evaluated as indicated in section 2. Results are expressed as cpm for the same number of cells obtained from a 100-mm culture dish. Numbers in parentheses represent percentages of FN or LN cpm counted in cell lysates and culture media. Each value represents the mean  $\pm$  S.D. of 4 separate experiments.

acquisition of enterocyte functions, which may be due to decreased gene transcription and/or mRNA stability

Quantitative analysis of FN and LN during differentiation of Caco-2 cells indicates that differentiated cells expressed significantly lower amounts of FN and LN than the non-differentiated ones. Interestingly, the ratio of amounts secreted to those synthesized was not modified throughout the differentiation process. Since decreases in FN and LN polypeptide synthesis are roughly accounted for by decreases in mRNA levels, one can suggest that the differentiation-induced down-regulation of FN and LN expression is mainly restricted to transcriptional and/or post-transcriptional levels. In this regard, evidence has now been provided that the genetic program of terminal differentiation is often, if not always, associated with repression of specific genes [31].

The signal transduction pathway involved in differentiation-induced FN and LN decreases is unknown. Recent data support a role for protein kinase C (PKC) in FN accumulation [32]. In view of our previous results [16,33] pointing to a regular decrease in the amount of active membrane-bound PKC throughout Caco-2 cell differentiation, one can wonder whether there is a relationship between reduced active PKC and FN and LN down-regulation during this process.

In conclusion, our results provide the first evidence that enterocytic differentiation of Caco-2 cells involves both transcriptional and/or post-transcriptional down-regulation of FN and LN gene expression.

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