Pancreatic trypsinogen I expression during cell growth and differentiation of two human colon carcinoma cells

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Bernard-Perrone, Françoise, Jacqueline Carrere, Wanda Renaud, Christine Moriscot, Karine Thoreux, Patrice Bernard, Alain Servin, Daniel Balas, and Françoise Senegas-Balas. Pancreatic trypsinogen I expression during cell growth and differentiation of two human colon carcinoma cells. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G1077–G1086, 1998.—Pancreatic trypsin has been found to induce tight junction or dome formation in some colon cancer cell lines (HT-29, Caco-2), and a tumor-associated trypsinogen, trypsinogen type II, has been isolated from another colon cancer cell line (COLO 205). We have tried to determine if trypsinogen is present and how its expression varies during cell culture in HT-29 Glc+/- and Caco-2 cells, which exhibit enteroctytic differentiation, and in HT-29 Glc+ cells, which never differentiate. Trypsinogen mRNA presence and expression were demonstrated in these cells by mRNA hybridization and immunoenzymatic assays. We have also examined whether trypsinogen is present in zymogen form in the medium. All results have been compared with those obtained in HT-29 Glc+ cells, which remain undifferentiated.

MATERIALS AND METHODS

Cell lines and culture conditions. Caco-2 and HT-29 cells derive from human colorectal adenocarcinoma (Dr. J. Fogh, Sloan Kettering Memorial Center, Rye, NY). As is well known (38), Caco-2 cells spontaneously differentiate after confluence. The following two populations of HT-29 cells were used in this study. 1) Parental HT-29 cells (referred to as HT-29 Glc+ cells) are grown in a medium containing glucose and remain essentially undifferentiated throughout the cell culture, with only 2% of postconfluent cells undergoing enteroctytic differentiation (23, 38). 2) Permanently differentiated HT-29 cells (referred to as HT-29 Glc+/- cells) were obtained from A. Zweibaum (Institut National de la Santé et de la Recherche Médicale, Villejuif, France). HT-29 Glc+/- cells are a 100% enterocytic subpopulation (18) obtained by selection through glucose deprivation that maintains its differentiation characteristics when switched back to standard glucose-containing medium (24). HT-29 Glc+/- cells also differentiate after confluence. Phase-contrast microscopy showed a dense and well-organized brush border, which carpets the apical surface, and with scanning electron microscopy the presence of uniformly distributed tall and regular microvilli was observed (3, 24). These cells expressed a low amount of aminopeptidase N and no sucrose (24). Another brush-border hydrolase, DPPIV, was found by cytoimmunologic technique in these cells after 20 days of culture (2, 24).

Cells were grown in DMEM (Sigma Chemical, St. Louis, MO) supplemented with 10% (HT-29) or 20% (Caco-2) inactivated (30 min at 56°C) FCS (GIBCO, Grand Island, NY) and 1% nonessential amino acids (Caco-2). Cells were seeded at 8 × 10⁴ cells/ml in 35-mm petri dishes or on glass coverslips, which were placed in six-well tissue culture plates (Corning Glass Works, Corning, NY). All the experiments and cell maintenance were carried out at 37°C in 10% CO₂-90% air. The culture medium was changed daily.

Growth curves were determined to define the exponential and stationary phases of growth and confluence. Four wells were carefully washed with PBS, and the attached cells were

tight junctions can be assembled in a variety of tissues and cultured cells by treatment of proteases (26, 27, 34, 44). In some colon cancer cell lines (parental HT-29 and Caco-2 cells), pancreatic trypsin, added to the culture cell medium, has been shown to be a potent inducer of tight junction (1, 9, 16, 33, 40, 46) and dome formation (HT-29 D4 cells) (11), which are characteristic of cell differentiation. Moreover, a trypsinogen type II isoenzyme, the tumor-associated trypsinogen II, has been isolated from the culture medium of another colon cancer cell line, COLO 205 (20).

We have tried to determine if trypsinogen is present in other colon cancer cell lines and if its expression is observed during a particular stage of cell differentiation.

Caco-2 and HT-29 Glc+/- cells exhibit a pattern of enteroctytic differentiation (2, 3, 18, 24, 38). This differentiation has been demonstrated by the appearance of tight junctions (Caco-2), well-organized brush borders, and intestinal hydrolases (Caco-2 and HT-29 Glc+/- cells). Thus we considered tight junctions and dipeptidyl-peptidase IV (DPPIV)- and sucrase-specific activities to be markers of differentiation. We examined Caco-2 and HT-29 Glc+/- cell lines for the presence of trypsinogen by cytoimmunofluorescence, Western blot analysis, and RT-PCR. Trypsinogen expression variations were assessed in both cell homogenate and culture media by mRNA hybridization and immunoenzymatic assays. We have also examined whether trypsinogen is present in zymogen form in the medium. All results have been compared with those obtained in HT-29 Glc+ cells, which remain undifferentiated.
then removed with trypsin-EDTA (0.15%/0.1%) and counted in the presence of trypan blue using a Malassez counting cell.

Preparation of proteins and antibodies. Human trypsin type I was prepared by autoactivation of partially purified human pancreatic trypsinogen I. DP-trypsin I was obtained by incubating purified trypsin I with disopropyl fluorophosphosphate at pH 7.8 (24 h). Antiserum against DP-trypsin I was raised in rabbits (kindly provided by O. Guy-Crotte, Groupe de Recherche sur les Glandes Excirimes, Marseille, France). An antisera against human trypsinogen I was also prepared by injecting the 23-kDa band isolated by SDS-gel electrophoresis of the partially purified zymogen (kindly provided by O. Guy-Crotte). The antibodies tested by Western blot analysis against human pancreatic juice revealed both trypsinogens I and II (13).

To avoid false-positive reactions resulting from the rabbit blood groups, we ascertained that antibodies against human erythrocytes were absent from the two rabbit sera used (35).

Antiserum against human ZO-1, which is associated with the cytoplasmic face of the tight junction, was supplied by Zymed Laboratories (San Francisco, CA). Antiserum was raised in rabbits against a 69-kDa fusion protein, corresponding to amino acids 463–1109 of human ZO-1 cDNA (48). This antibody will react with ZO-1 in cell lysates of p55 was observed, and the antibody reacted with human, mouse, rat, guinea pig, and canine ZO-1 on Western blot. ZO-1 was immunoprecipitated from Caco-2 cells.

Immunofluorescence staining. Indirect immunofluorescence was performed on cell monolayers (Caco-2, HT-29 Glc+, and HT-29 Glc+/− cells) grown on glass coverslips. Cells were permeabilized in saponin (0.1%) for 10 min and rinsed in 0.05 M phosphate buffer with 8.5 g/l of NaCl (pH 7.2) (PBS).

Cells were incubated sequentially with 3% nonimmune goat serum, primary rabbit antibodies against human trypsinogen I or ZO-1 (1:20 to 1:100 in PBS, 3% goat serum, 0.2% gelatin, for 12 h at 4°C) and an FITC-conjugated goat anti-rabbit IgG (1:100, 1 h) (Dako).

HT-29 Glc+/− cells (day 3) were also observed with the use of a confocal laser microscope (LSM 410; Carl Zeiss, Iena, Germany) after using primary rabbit antibodies against human trypsinogen I. An argon ion laser adjusted at 488 nm was used for the analysis of fluorescence. Optical sectioning was used to collect four horizontal views (5 μm).

The following controls were performed. 1) The primary antibodies were replaced with PBS or preimmune rabbit serum. 2) FITC-conjugated goat anti-rabbit IgG was replaced with PBS. 3) Immunoadsorption tests were also performed by incubating 0.2 mg/ml of human DP-trypsin I (at 4°C for 24 h) with the antiserum against human trypsinogen I.

Preparation of cell homogenates and cell culture media. The cell culture media (Caco-2, HT-29 Glc+, and HT-29 Glc+/− cells) were harvested with phenylmethylsulfonyl fluoride (PMSF; 1 mM), lyophilized, and stored at 80°C. The following controls were performed.

Activation by enterokinase. The fraction corresponding to the highest IRT for HT-29 at day 10 for Glc+/− cells was filtrated on 0.22-μm filter units (Millipore, Mollessheim, France). We exposed 200 µl of this IRT solution (6 ng of IRT) to enterokinase (in excess amount) in Tris·HCl buffer containing 200 mM NaCl (pH 7.6, 4°C). Column calibration was made with the following reference proteins of Nase A (relative molecular weight, M1 13,700), α-chymotrypsinogen A (M, 25,000), ovalbumin (M, 43,000), and BSA (M, 67,000) (Pharmacia Fine Chemicals). Loaded samples were 500 µl, and collected fractions were 2 ml with an elution rate of 3 ml/h. Aliquots of 50 µl of each fraction were assayed twice by immunobassay.

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Brush-border hydrolase assays. Sucrase (EC 3.2.1.48) activity was measured according to the method of Dahlqvist (10) (3 dishes/cell line/experimental time). The results of each cell homogenate were expressed as nanomoles of substrate hydrolyzed per minute at 37°C (mIU) per milligram of protein.

Enzyme immunoassay of trypsinogen I. Human trypsinogen I immunoreactivity (IRT) was measured by a noncompetitive "sandwich" enzyme immunoassay using the antibody against human DP-trypsin I in cell culture media and in cell homogenates (4 dishes/cell line/experimental time) (5). It was verified that this immunoassay does not recognize purified human pancreatic trypsinogen I or trypsin from other species (28).

The results in each cell homogenate (3 assays/homogenate) were expressed per microgram of protein measured in this cell line homogenate. The IRT level in cell lysate was also assessed with respect to the cell number for each petri dish. After the IRT levels in each cell medium were measured, the quantity of IRT found was divided by the amount of cell homogenate proteins assayed in a dish (3 assays/medium). The amount of trypsinogen I released in the culture medium by 1 μg of cell homogenate protein could thus be compared with the quantity synthesized by 1 μg of cell homogenate protein.

Gel filtration experiments. The molecular size distribution of IRT found in the three cell line culture media (day 10) was determined by Sephadex G100 SF filtration in a 50 mM Tris·HCl buffer containing 200 mM NaCl (pH 7.6, 4°C). Column calibration was made with the following reference proteins: Nase A (relative molecular weight, M1 13,700), α-chymotrypsinogen A (M, 25,000), ovalbumin (M, 43,000), and BSA (M, 67,000) (Pharmacia Fine Chemicals). Loaded samples were 500 µl, and collected fractions were 2 ml with an elution rate of 3 ml/h. Aliquots of 50 µl of each fraction were assayed twice by immunobassay.
Immobilized proteins were characterized by using the antibody against human trypsinogen I (1:250) in PBS containing 5% (wt/vol) dry skim milk or whole preimmune rabbit serum in the same conditions. The blots were incubated (1 h) with horseradish peroxidase-labeled anti-rabbit antibody (1:5,000; Bio-Rad, Hercules, CA). Then the membranes were washed three times with PBS, 0.1% (vol/vol) Tween 20. The immunoreactive bands were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The standard prestained proteins (Bio-Rad prestained SDS-PAGE standards; Hercules, CA) used were as follows: phosphorylase b (105 kDa), BSA (84 kDa), ovalbumin (49 kDa), carbonic anhydrase (33.3 kDa), soybean trypsin inhibitor (28.6 kDa), and lysozyme (19.4 kDa).

RT-PCR. Purification of mRNA from total RNA (Caco-2, HT-29 Glc+/−, and HT-29 Glc+/− cells homogenates at day 8) was performed with an mRNA separatory kit (Clontech Laboratories, Palo Alto, CA). mRNA were reverse transcribed into cDNA using oligo(dT)15 (Boehringer Mannheim) as primer, with or without SuperScript II RT (Life Technologies, Gaithersburg, MD) according to the manufacturer’s recommendation, and the preparation qualities were monitored by agarose gel electrophoresis. PCR amplifications were accomplished (Taq DNA polymerase; Life Technologies) with an MJ Research, PT-100 DNA Thermal Cycler as suggested by the manufacturer. The RNA were used for future trypsinogen mRNA PCR analysis only if human actin amplification was performed with an mRNA separator kit (Clontech Laboratories, Palo Alto, CA). mRNA were reverse transcribed into cDNA using oligo(dT)15 (Boehringer Mannheim) as primer, with or without SuperScript II RT (Life Technologies, Gaithersburg, MD) according to the manufacturer’s recommendation, and the preparation qualities were monitored by agarose gel electrophoresis. PCR amplifications were accomplished (Taq DNA polymerase; Life Technologies) with an MJ Research, PT-100 DNA Thermal Cycler as suggested by the manufacturer. The mRNA were used for future trypsinogen mRNA PCR analysis only if human actin amplification was observed and none were used without the RT indicating the absence of DNA contamination. The sense primer was 5′-GCGACTGCTGGGCCCCGTGCCC-3′ (nt 390–414), and the antisense primer was 5′-GGACGCTGCTTCTGGTGCCTGTGCCTGTGCC-3′ (nt 36–57). Samples from 0.5 µg of cell RNA and 1 µg of human total pancreatic RNA were initially denatured at 95°C for 1 min. Cycling parameters (35 cycles) were as follows: denaturation at 95°C for 1 min, annealing at 69°C for 1 min, and extension at 72°C for 1 min. A final extension at 72°C for 5 min was performed. Portions of the amplified products were analyzed by agarose gel electrophoresis (1.5%) in the presence of ethidium bromide (for 1 h at 100 V).

Quantitative analysis of trypsinogen mRNAs by dot-blot hybridization. After being washed twice in PBS, the Caco-2, HT-29 Glc−, and HT-29 Glc+/− cells were scraped and dispersed in 4 M guanidinium isothiocyanate (3 dishes/cell line/experimental time). Human pancreas (obtained from organ donor) and the HL-60 cell line were used as positive and negative controls, respectively (29). Total RNAs were purified according to the method of Chirgwin et al. (8). The preparation qualities were monitored by agarose gel electrophoresis in the presence of formaldehyde.

Human pancreatic trypsinogen cDNA was produced by RT-PCR from 1 µg of human pancreatic RNA by using the sense and antisense primers previously used for PCR (29). The trypsinogen insert length was 379 bp. This cDNA can hybridize with trypsinogen I and II mRNAs. Its specificity was checked by Northern blot on total RNA, purified from a pancreas obtained from an organ donor. A single band of the expected size (0.9 kb) was observed. This cDNA was labeled by "nick-translation" with [32P]dCTP.

Dot-blot assays were performed according to the techniques of White and Bancroft (47) and Favarolo et al. (12). Sequential dilutions (5–0.156 µg for the cells and 0.5–0.0156 µg for pancreatic tissue) were loaded onto nitrocellulose membrane (3 assays). mRNA transcript levels were quantified by dot-blot analysis and densitometry after hybridization with the trypsinogen probe. The slopes, calculated by least-square regression analysis, gave an estimate of the relative amount of trypsinogen mRNA in total RNA. mRNA concentrations were expressed in arbitrary units per microgram of total RNA. The regression coefficient was always >0.98.

Statistical methods. IRT and mRNA levels and hydrodase specific activities were expressed as means ± SE. Statistical analysis was made with ANOVA followed by Scheffe's multiple comparison test using Stat View software (Brain Power, Calabasas, CA).

RESULTS

Cell growth. Cell confluence was reached between days 5 and 6 for HT-29 Glc+ and HT-29 Glc+/− cells and between days 6 and 7 for Caco-2 cells. A stationary phase was noted at days 11, 12, and 13 for HT-29 Glc+, HT-29 Glc+/−, and Caco-2 cells, respectively. Figure 1 depicts the growth profile of the cell cultures.

DPPIV and sucrase activities. Sucrase and DPPIV activities were demonstrated after day 5 in Caco-2 cells and then increased as expected (15, 38) (Fig. 2). In HT-29 Glc+ cells, very weak DPPIV specific activity was detected, as previously described (23). In HT-29 Glc+/− cells, DPPIV activity was shown at day 6, confirming that enterocyte differentiation occurs under confluence. This activity increased significantly in HT-29 Glc+/− cells from day 6 to days 15–16; at this time, the confluent cells showed a well-organized brush border (2, 24). Afterward, these activities moderately increased. Our assay confirmed previous cytoimmunologic results with an anti-DPPIV monoclonal antibody applied to HT-29 Glc+/− cells after day 20 (2, 24). DPPIV activity was lower in HT-29 Glc+/− cells than in Caco-2 cells.

Cytoimmunofluorescence demonstration of ZO-1 in cell cultures (day 10). Gasketlike labeling, typical of tight junctions, is clearly evident on HT-29 Glc+/− and Caco-2 cell monolayers with anti-ZO-1 antiserum, demonstrating that these cells differentiated (Fig. 3).

Cytoimmunofluorescence demonstration of trypsinogen in cell cultures. Caco-2, HT-29 Glc+, and HT-29 Glc+/− cell cytoplasms exhibited diffuse staining when exposed to anti-human trypsinogen I antibody at days 3, 7, 10, and 21 (Fig. 4). However, striking differences in apparent trypsinogen expression were revealed when
we observed the same coverslip, because the labeling intensity varied from one group of cells to another (Fig. 4). This is consistent with the fact that Caco-2, HT-29 Glc+, and HT-29 Glc+/- cells are not clonal. However, these variations could also reflect different levels of trypsinogen synthesis or secretion.

Fig. 2. Variation of specific activities of intestinal brush-border hydrolases [dipeptidyl-peptidase IV (DPPIV) and sucrase] in HT-29 Glc+/-, HT-29 Glc+, and Caco-2 cell homogenates over the 21 days of culture (4 dishes/cell line/experimental time). For each homogenate, results were expressed as nmol of substrate hydrolyzed/min (mIU) and assessed with respect to the protein level in this homogenate. Values are means ± SEM of 4 determinations. For HT-29 Glc+/- and Caco-2 cells, these hydrolase activities increased after day 5, demonstrating that these cells differentiated. *P < 0.05, **P < 0.01, significantly different from day 3. Top: HT-29 Glc+/-; O, HT-29 Glc+; △, Caco-2. Bottom: sucrase activities were only measured in Caco-2 cells (△).

Front views of fixed HT-29 Glc+/- cell monolayers showed that labeling could be observed in all the cell cytoplasms (Fig. 5).

All the control tests were negative. Cell monolayers treated with the antitrypsinogen previously adsorbed with human DP-trypsin I showed negative results, demonstrating that the labeling observed with the anti-human trypsinogen I antibody was specific (Fig. 4F).

Demonstration of IRT. IRT levels expressed in homogenate protein contents did not statistically vary in HT-29 Glc+ cells during the cell culture (Fig. 6A) and decreased with respect to the cell number (Fig. 6C). No variation of IRT levels in cell homogenate could be seen between days 3 and 5 for HT-29 Glc+/- cells (Table 1). In the HT-29 Glc+/- and Caco-2 cell homogenates, IRT levels were much higher at day 10 than at days 3 and 5 (HT-29 Glc+/- cells, +325%; Caco-2 cells, +147%) (Fig. 6, A-D). They strongly decreased from days 10 to 13 (HT-29 Glc+/- cells, −34%; Caco-2 cells, −94%) (Fig. 6, A-D). This decrease was particularly strong in Caco-2 cells in which the levels fell to much lower than those observed at day 3. In HT-29 Glc+/- cells, IRT levels at day 13 remained higher than those at days 3 and 5; between days 13 and 21, IRT levels did not vary (Fig. 6, A-D). The highest levels were observed for HT-29 Glc+/- cells.

IRT levels were also characterized in all cell culture media (Fig. 6, E and F). They varied according to the IRT variation patterns in cell homogenates. The amount of released IRT was much smaller than in cell homogenates. Moreover, the variations in cell culture media were smaller than those observed in cell homogenates, especially in HT-29 Glc+/- cells (day 10, +60%; day 13, +25%; day 21, no increase in release).

Molecular size distribution of IRT in cell culture media. Identical profiles of IRT molecular size distribution were observed for the three cell line culture media. Typical profiles of HT-29 Glc+/- and Caco-2 cells are presented in Fig. 7. Fractions containing IRT were eluted at the end of the second void volume with the 25-kDa proteins. This elution profile is consistent with the presence of trypsinogen (24 kDa) in the cell culture media. If active trypsin had been present, it would have
been mainly eluted in the first chromatographic fractions as complexes associated with serum trypsin-α1-proteinase inhibitor. Because cell culture media were treated in the same conditions for all the cell lines, these gel filtrations confirmed that the quantity of IRT released was smaller in Caco-2 and HT-29 Glc± cells than in HT-29 Glc+/− cells.

When HT-29 Glc+/− gel filtration fractions were applied on gelatin, no release of India ink was observed with the fraction of IRT devoid of enterokinase. In contrast, India ink was spread out in the fraction previously incubated with enterokinase (Fig. 8), demonstrating that gelatin was digested and thus that the IRT present in HT-29 Glc+/− cell culture medium was

Fig. 4. Demonstration of trypsinogen presence in Caco-2, HT-29 Glc+, and HT-29 Glc+/− cell lines using immunocytofluorescence. Paraformaldehyde-fixed and saponin-treated cell monolayers were exposed to rabbit anti-human trypsinogen I antiserum and FITC-conjugated goat anti-rabbit IgG. Strong labeling could be seen in all cells on all days. However, it should be noted that labeling intensity varied from 1 group of cells to another. A: HT-29 Glc+/− cells at day 3. B: HT-29 Glc+/− cells at day 7. C: HT-29 Glc+/− cells at day 21. D: Caco-2 cells at day 3. E: HT-29 Glc+ cells at day 10. F: HT-29 Glc+/− cells at day 7; cell monolayers were treated with antiserum previously adsorbed with DP-trypsin I and showed negative results. A–C and E: bar, 6 µm. D: bar, 13 µm.

Fig. 5. Front-facing views of immunolocalization of trypsinogen by laser-confocal microscopy of HT-29 Glc+/− cell line (day 3). Images shown are from the apical part of the cells (A) to the basal end of the cells (D); they are separated by 5 µm. Labeling is uniformly distributed throughout the cell cytoplasm area.
in the form of trypsinogen that was activated in trypsin by enterokinase. Western immunoblot of cell homogenates. Western blot analysis demonstrated two bands of 25 and 23 kDa, corresponding to trypsinogens I and II, respectively, in nonactivated human pancreatic juice. In the cell homogenates of HT-29 Glc+/-, HT-29 Glc+, and Caco-2 cells, we observed only a band corresponding to trypsinogen I that is consistent with the presence of only trypsinogen I in these cells (Fig. 9). These blots confirmed that the quantity of trypsinogen is greater in HT-29 Glc+/− cells than in HT-29 Glc+ cells and greater even than in Caco-2 cells. No band could be seen with preimmune serum.

RT-PCR. PCR analysis of cDNA samples generated from the three cell lines indicated the presence of a specific trypsinogen signal with the expected length (379 bp) (Fig. 10, lanes C, E, and G). The product observed was identical to the one demonstrated in human pancreas used as a positive control (Fig. 10, lane I). Cross-tissue contamination could be excluded because no RT-PCR product was detected when primers were used alone in liquid controls (Fig. 10, lanes B, D, F, H, and J). Trypsinogen mRNA presence was thus demonstrated in the three colon cancer cell lines.

Quantitative analysis of trypsinogen mRNAs by dot-blot hybridization. Dot-blot assays confirmed that trypsinogen mRNA was expressed by HT-29 Glc+/-, HT-29 Glc+, and Caco-2 cell lines as in human pancreatic tissue (positive control) (Fig. 11). As expected, there are ~100 times less trypsinogen mRNA in colon cancer cells than in pancreatic tissue, and no hybridization was observed for the HL-60 cell line (negative control). The relative amount of trypsinogen mRNA in total RNA with respect to the successive days of culture is presented in Fig. 12. The relative quantification of trypsinogen mRNA shows that similar amounts of trypsinogen mRNA in the three colon cancer cell lines are present at day 3 and that the amount in Caco-2 cells was greater than in HT-29 Glc+/- cells at day 10. Trypsinogen mRNA expression did not vary in HT-29 Glc+ cells. HT-29 Glc+/− and Caco-2 cell lines exhibited a similar pattern of trypsinogen mRNA expression during cell culture. A strong increase at day 10 (HT-29 Glc+/− cells, +107%; Caco-2 cells, +347%) was followed at day 13 by a strong decrease to the values observed at day 3. Between day 13 and day 21 these mRNA expressions did not vary.

**DISCUSSION**

The presence of trypsinogen in Caco-2, HT-29 Glc+/-, and HT-29 Glc+ cells was clearly shown over the 21 days of culture. Staining in colon cancer cells was seen with the antibody against human trypsinogen I, and trypsinogen mRNA expression was demonstrated until day 21. The expression of trypsinogen in these cells was also confirmed by RT-PCR techniques, Western immunoblot analysis, gel filtration, and digestion of gelatin. Our immunoassay could only recognize trypsinogen I (28). Moreover, we demonstrated through Western immunoblot analysis that these cells expressed only trypsinogen I, whereas in another colon cancer cell line (COLO 205), trypsinogen II is mainly expressed (20). In addition, we showed that trypsinogen was released in culture medium.

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**Table 1. Kinetics of IRT in cell homogenates**

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<thead>
<tr>
<th>Time (days)</th>
<th>HT-29 Glc+/-</th>
<th>HT-29 Glc+</th>
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<tr>
<td>Day 3</td>
<td>58 ± 14.3</td>
<td>11.4 ± 2.6</td>
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<tr>
<td>Day 5</td>
<td>55 ± 11.9</td>
<td>11.5 ± 2.4</td>
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<tr>
<td>Day 10</td>
<td>247 ± 11.7*</td>
<td>12.1 ± 2.0</td>
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<tr>
<td>Day 13</td>
<td>189 ± 12.9†</td>
<td>10.3 ± 1.8</td>
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<tr>
<td>Day 15</td>
<td>174 ± 9.5†</td>
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Immunoreactive trypsinogen (IRT) in cell lysates [in ng/µg protein] during cell growth of HT-29 Glc+ and HT-29 Glc+/- cell lines. *P < 0.001, significantly different from day 3, †P < 0.002, significantly different from day 3. NS, not significant.
IRT levels in cell homogenates and culture media and mRNA expression presented similar patterns of variation over the period studied for each cell line. However, IRT levels were strikingly higher in HT-29 Glc+/- cells than in Caco-2 cells, although the relative amounts of trypsinogen mRNA were similar in the two cell lines at day 3 and higher in Caco-2 cells at day 10. These results imply trypsinogen mRNA posttranscriptional regulation in Caco-2 cells. Moreover, the decrease in IRT level in cell homogenate after the 10th day was not as rapid as the decrease in mRNA levels in HT-29 Glc+/- cells. This slowdown can be accounted for by the fact that the amount of trypsinogen released was lower than the amount synthesized and thus trypsinogen is stored in the cells.

Nevertheless, the pattern of IRT variation and trypsinogen mRNA expression was different in cells undergoing differentiation (HT-29 Glc+/- and Caco-2 cells) and in permanently undifferentiated cells (HT-29 Glc+ cells). No significant variation could be seen in HT-29 Glc+ cells when a strong increase in IRT levels and trypsin mRNA expression was observed in HT-29 Glc+/- and Caco-2 cells at day 10.

The differentiation of these cancer cell lines appeared (after day 5), when proliferation could still be observed, and lasted until the beginning of the stationary phase of growth, as is the case in the small intestine crypt proliferative compartment, where differentiating cells are also known to divide (7). In HT-29 Glc+/- and in Caco-2 cells, the increase in IRT levels and trypsin mRNA expression might be associated with cellular proliferation since growth could still be observed at day 10. However, no variation in these parameters could be observed in HT-29 Glc+/- cells between days 3 and 5.

**Fig. 7.** Identification of molecular forms of IRT by gel filtration on Sephadex G-100SF. The representative profiles obtained with HT-29 Glc+/- (A) and Caco-2 cell (B) culture media at day 10 show that fractions containing IRT eluted with 25-kDa proteins. Column, 1.5 x 90 cm; load, 500 µl of supernatant; fraction volume, 2 ml. V₀ is the void of the column of gel filtration. It represents the volume of liquid present in the column outside the gel. It is measured by the elution volume of proteins of high molecular weight that do not enter the gel. V₁ is 2 × V₀.

**Fig. 8.** Demonstration that IRT was trypsinogen. The fraction corresponding to the highest IRT for HT-29 Glc+/- cells (day 10) (6 ng of immunoreactive trypsinogen) was exposed to enterokinase (in excess) at 4°C (2 h). This solution was loaded on Kohn gelatin (24 h, 37°C) into which india ink was incorporated (A). The result was compared with results obtained without enterokinase in the same conditions (B). No release of India ink was observed without enterokinase. In contrast, India ink spread into the solution with enterokinase (A), demonstrating that gelatin was digested and thus that the IRT present in HT-29 Glc+/- cell culture medium was trypsinogen that was activated in trypsin by enterokinase.

**Fig. 9.** Representative Western immunoblots of trypsinogen isolated from Caco-2 (lane 1), HT-29 Glc+/- (lane 2), and HT-29 Glc+ cell (lane 3) lysates (60, 20, and 40 µg of proteins, respectively) (day 5), using rabbit anti-human trypsinogen I antibody. As positive control, immunoblots were also performed with nonactivated human pancreatic juice (lane 4, 0.1 µg of proteins). As negative controls, Western immunoblots were also performed simultaneously with the same lysates of Caco-2 (lane 5), HT-29 Glc+/- (lane 6), and HT-29 Glc+ cells (lane 7) and pancreas (lane 8) by using preimmune rabbit serum as primary antibody. The immunoreactive bands were detected by chemiluminescence. Trypsinogens I and II were seen in human pancreatic juice, whereas only trypsinogen I was demonstrated in cancer colon cell lines.
corresponding to the exponential phase of growth (Tables 1 and 2). In addition, IRT levels were higher during the stationary phase of growth of HT-29 Glc+/- cells (days 12-20) than at days 3 or 5 (Tables 1 and 2). Moreover, the greatest increase in cell proliferation was observed in HT-29 Glc+ cells, and no variation of their trypsinogen mRNA and protein expression was demonstrated (Tables 1 and 2).

It is well known that the burst of differentiation in Caco-2 cells is characterized first by the appearance of tight junctions inducing cell polarity and afterward, by those of microvilli and brush-border hydrolase activities (15, 38). The presence of well-formed brush borders and DPPIV activities was also demonstrated in HT-29 Glc+/– cells after confluence (23). We confirmed these results by the assay of sucrase and DPPIV and the presence of tight junctions, demonstrated for the first time in HT-29 Glc+/- cells. The increase in IRT levels and trypsinogen mRNA expression was observed at day 10 in Caco-2 and HT-29 Glc+/- cells and might instead be associated with the first steps of cell differentiation. This hypothesis also corresponds well with the fact that no increase in IRT level and trypsinogen mRNA expression was seen in nondifferentiating HT-29 Glc+ cells. These results also agree with studies demonstrating that trypsin added in differentiating HT-29 D4 cell culture medium induced dome formation (11), which is considered to be a functional differentiation (25). Moreover, these results were in line with those demonstrating that trypsin added in nondifferentiating HT-29 Glc+ cell culture medium induced the formation of tight junction strands (16, 40). In addition, serine protease inhibitors, leupeptin, and antipain inhibited cesium sulfate-induced tight junction strand formation in HT-29 Glc+ and Caco-2 cells, and the formation of tight junction strands after the removal of leupeptin was not affected by cycloheximide (1). It was suggested that tight junction fibrils are assembled from protein precursors in the cell membrane and that limited proteolysis by an endogenous cell membrane protease is required to transform these proteins into functional elements of the tight junction. They also suggested that this protease activity might be nonfunctional in HT-29 Glc+ cells and might be substituted by trypsin added to the medium. We demonstrated that trypsinogen was released in HT-29 Glc+/- cell medium at a rate of 500 ng/ml at day 10, which is a dose similar to the quantity of trypsin added in all the experiments showing induction of tight junctions. In addition, the staining with

<table>
<thead>
<tr>
<th>Table 2. Number of cells during cell growth</th>
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<tbody>
<tr>
<td>HT-29 Glc+/-</td>
<td>P</td>
<td>HT-29 Glc+</td>
</tr>
<tr>
<td>Day 3</td>
<td>1,633 ± 78</td>
<td>2,300 ± 95*</td>
</tr>
<tr>
<td>Day 4</td>
<td>4,667 ± 159</td>
<td>7,187 ± 204*</td>
</tr>
<tr>
<td>Day 5</td>
<td>11,230 ± 275</td>
<td>15,630 ± 217*</td>
</tr>
<tr>
<td>Day 9</td>
<td>33,730 ± 112</td>
<td>33,570 ± 140</td>
</tr>
<tr>
<td>Day 10</td>
<td>36,270 ± 855</td>
<td>41,000 ± 938*</td>
</tr>
<tr>
<td>Day 11</td>
<td>38,730 ± 927</td>
<td>46,800 ± 123*</td>
</tr>
<tr>
<td>Day 12</td>
<td>43,500 ± 670</td>
<td>46,200 ± 132*</td>
</tr>
<tr>
<td>Day 13</td>
<td>42,000 ± 720</td>
<td>47,000 ± 208*</td>
</tr>
</tbody>
</table>

| Variation of cell number [no. of cells/ml (× 10^4)] during growth of HT-29 Glc+ and HT-29 Glc+/- cell lines. P values refer to significant difference between results observed on consecutive days. *P < 0.001, significantly different from HT-29 Glc+/- cell line at the same day of culture.
ZO-1 antiserum at day 10 showed a large amount of tight junctions. At this time, many differentiating cells were present (Table 1), requiring a large quantity of tight junction inducer, thus concording with the peak trypsinogen level observed at day 10.

Nevertheless, this hypothesis could be contested, because in our study trypsinogen remained mainly in zymogen form in the culture medium. This is the case with many proteases in cancer cells: trypsinogen I or I1 or related protein in pancreatic cancer cell lines (Capan-1 and CFPAC-1 cells), in pancreatic ductal adenocarcinoma, in cyst fluid of mucinous ovarian tumors, in COLO 205 colon cancer cell line, in gastric adenocarcinoma, in K-562 erythroleukemia, and in HT-1080 fibrocarcinoma cell lines (19, 21, 28, 32) and pepsinogen C in breast tumors (41). In addition, procathepsin B was found in the culture medium of several human colon carcinoma cells (Caco-2, HT-29, and COLO 205 cell lines) (17). It should be noted that in tumor invasion, invading cells utilize integral membrane proteases to cleave and activate secreted metalloproteinases or serine proteases on the tumor cell surface (6, 42). We hypothesize that trypsinogen I was activated by a protease on the surface of HT-29 Glc+/- cells and that trypsin could induce tight junction formation and cell differentiation. This hypothesis agrees with the presence and release of trypsinogen by Paneth cells (43). These cells are located in the bottom of small intestinal crypts, in close contact with stem cells, and the presence of trypsinogen could trigger the differentiation of the immature cells.

Another interesting finding in this study was that trypsinogen expression was revealed in cancer colon cell lines, as in many benign or malignant tumor cells, when it could not be demonstrated in the corresponding healthy tissue (4, 28, 32). Moreover, trypsinogen levels appeared to correlate with the degree of malignancy in ovarian tumors (19). In addition, pancreatic-secretory trypsin inhibitor, also referred to as tumor-associated trypsin inhibitor, was considered to be a marker of pancreatic cancer (14, 31, 36, 37, 45). It may be assumed that trypsinogen expression in the human colon carcinoma cell lines that we studied could be associated with benign or malignant processes in the colon.

HT-29 Glc+/-, HT-29 Glc+, and Caco-2 cells expressed trypsinogen mRNA and synthesized trypsinogen I and released it mainly in nonactivated form in the culture media over the 21 days studied. However, the trypsinogen expression profiles during the culture were not the same in cells eliciting differentiation as in cells that remain undifferentiated. In differentiated cells, the peak in trypsinogen I expression was observed during the first stages of differentiation.

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