New Methodological Approach to Induce a Differentiation Phenotype in Caco-2 Cells Prior to Post-confluence Stage

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Abstract. *Background: Various differentiation-inducing agents or harvesting of spontaneously late post-confluence cultures have been used to differentiate the human colon carcinoma Caco-2 cell line. We report a new procedure to generate pre-confluent subcultures of Caco-2 population at various stages of differentiation without altering culture conditions. Materials and Methods: Ultrastructural analysis, cell proliferation activity and biochemical markers of differentiation were evaluated at different passages. Results: Subcultures of Caco-2 cells at pre-confluence, exhibiting progressive acquisition of a more benign differentiation phenotype, were generated. Early passages of Caco-2 cells showed a well-developed brush border and incomplete junctional apparatus; subsequent subcultures yielded cell populations with well-developed junctions similar to those of small intestinal cells. Conclusion: These culture conditions represent a new versatile model not only to progressively induce the differentiation program in Caco-2 cells at pre-confluence without changes of culture media, but also to explore mechanistic modes of drug transport and tumor development.*

The failure to establish long-term primary culture of normal small intestinal and colon cells has led to the generation of a large panel of cell lines derived from gastrointestinal tumors (1). These cell lines are extensively used in *in vitro* and *in vivo* studies to gain insight into mechanisms underlying cancer development and for screening and characterization of agents able to induce a more benign or differentiated phenotype in neoplastic cells. In this respect, the human colon adenocarcinoma Caco-2 cell line has provided a wealth of information. These cell lines were established with the aim of studying cancer and anticancer therapies owing to their ability to induce tumors when inoculated into rodents. Subsequently, Caco-2 cells were defined as a cell population characterized by the ability to spontaneously undergo the differentiation process toward a "normal" intestinal phenotype in long-term culture after post-confluence (2), in contrast to HT-29 cells, which need variable inducers of cell differentiation in their medium (3). Subsequently, also for Caco-2 cells, the possibility of inducing a functional and morphological differentiation process, spontaneously, by means of specific culture conditions, or by using variable filter supports, was worked out (4-6). Thus, both at post-confluence and under experimental conditions employing differentiation agents, Caco-2 cells form a polarized monolayer characterized by the presence of domes, due to the existence of unidirectional fluxes of ions and water, and express specific markers such as the brush border enzyme sucrase-isomaltase and alkaline phosphatase. More recently, the validity of differentiated Caco-2 cells as a versatile model of the intestinal epithelium was strengthened by the recognition of their barrier properties. Differentiated, post-confluent Caco-2 cells exhibit a tight monolayer of mainly absorptive cells through their tight junctions (7) and express specific intestinal transporters for sugars, amino acids, oligopeptides, bile acids, vitamins and micronutrients (8). A limiting factor of these studies, however, is that apart from the coexistence of enterocytic and colon cells in a given Caco-2 cell population (9), most culture conditions adapted to induce differentiation in these cells, including long-term culture at post-confluence as well as the growth on special supports, are not representative of physiological conditions. We report here the possibility of generating a population of Caco-2 cells at different stages of differentiation, without altering the culture conditions and without reaching post-confluence.

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Materials and Methods

Cell culture media and all other reagents were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise. Fetal bovine serum was from EuroClone Ltd (West Yorkshire, UK).

Cell culture. The human colon carcinoma Caco-2 cell line (BS TCL 87) was obtained from the Istituto Zooprofilattico Sperimentale di Brescia (Brescia, Italy). Cells were routinely grown in 75 cm^2 plastic flasks (Costar, Concorezzo, Italy) in Eagle's minimum essential medium in Earle' Basal Salt Solution, supplemented with 15% fetal calf serum, 2 mM L-glutamine, 0.1 mg/L streptomycin, 100,000 U/L penicillin, 0.25 mg/L amphotericin-B and 1 mM sodium pyruvate. Cultures, kept at 37˚C in a 5% CO_2/ 95% air atmosphere, were periodically checked for the presence of mycoplasma and were found to be free of contamination.

Cell growth and differentiation conditions. The cell growth conditions were carefully standardized so as to provide good reproducibility and a routine procedure that allowed a high degree of cell differentiation to be reached. Caco-2 parental cells were seeded at $1x10^5$ cells/cm^2 in 75 cm^2 plastic flasks. The medium was changed twice a week. When cells reached 100% confluence, as checked by phase-contrast microscopy (Olympus, IX50, Tokyo, Japan), they were trypsinized after one day post-confluence (Trypsin-EDTA 0.05%-0. 02%), diluted, usually 1:3, and then seeded again in a new plastic flask. Each trypsinization and successive dilution in a new flask was considered as a cell passage. In this work the parental cell line from a single flask was cultured for 50 passages following the experimental procedure described and represented in a schematic diagram in Figure 1. At the indicated passage in culture, cells were plated in plastic flasks or petri dishes and grown till 80-90% confluence for further assays.

Isolation of brush border fraction. For the detection of alkaline phosphatase and sucrase-isomaltase activity, cells at different passages were seeded in 75 cm^2 flask and when 80-90% confluence was reached they were harvested in ice-cold physiological saline, washed three times and pellets obtained after centrifugation were stored at –80˚C. Cell extracts were prepared as described elsewhere previously (10-11) with minor modifications. Briefly, cell pellets (6-7 mg of proteins, determined following the Lowry assay) were homogenized with 1 ml ice-cold Tris/mannitol buffer (2 mM Tris, mannitol 50 mM, pH 7.1) and disrupted by ultrasonication. $CaCl_2$ was then added to the homogenate to a final concentration of 20 mM and the homogenate was placed at 4˚C for 10 min with frequent mixing on a rotating plate. After this incubation period, centrifugation (10 min, 950 xg, 4˚C) provided a supernatant that was subsequently centrifuged (30 min, 33500 xg, 4˚C) to yield a small pellet (fraction P2) containing the brush border membranes. This pellet was finally resuspended with a small aliquot of Tris/Mannitol buffer in order to make the assays of enzyme activities and the assay of protein content.

Alkaline phosphatase assay. The method previously described (12) and based on *p*-nitrophenol (PNP) production starting from *p*-nitrophenyl-phosphate (PNPP) was used in our series of experiments. P2 fractions of 20-50 μg were resuspended to a final volume of 50 μl. The reaction mixture consisting of 7 mM PNPP, 0.1 M sodium bicarbonate and 5 mM $MgCl_2$ in a volume of 0.5 ml

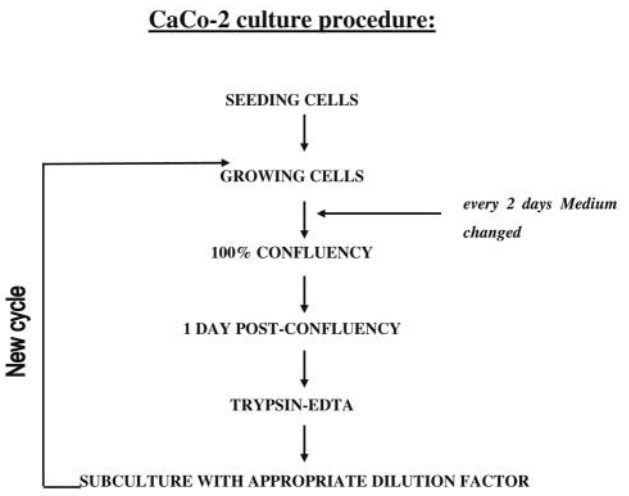

Figure 1. *Schematic diagram of the standardized Caco-2 cell culture methodology used.*

was prepared and added to all samples. Standard solutions of PNP ranging from 0 to 50 nmoles were prepared and diluted to 0.5 ml with the reaction mixture devoid of PNPP. After an incubation period of 25 min at 37˚C in the dark, the reaction was stopped by adding 1 ml of 0.1N NaOH to each tube. The absorbance was measured at 410 nm and enzyme activities were calculated according to a standard curve and expressed as mU/mg protein; 1 Unit is defined as the enzyme activity that hydrolyses 1 μmole of substrate per min. The protein content was measured following the Lowry method.

Sucrase-isomaltase assay. The one-step ultramicromethod was used without modifications (13). The P2 fractions (about 20 μg) were resuspended to a final volume of 20 μl in each tube. The glucose standard curve ranged from 0 to 50 μg of glucose and absorbance was read at 540 nm. The protein content was determined as reported above. Results are expressed as mU/mg protein enzyme, unit activity as defined in the preceding section.

Cell proliferation assay. The cells at different passages were cultured in standard medium at $1x10^4$ cells/well in a Microtiter plate (96-well, Greiner bio-one, Cellstar, Germany). Cell protein amount was determined using the chemiluminescent immunoassay kit based on the measurement of Bromodeoxyuridine (BrdU) incorporation during DNA synthesis (Roche Applied Science, Basel, Switzerland) as described by the manufacturer. Cell proliferation activity was measured after a 2 h pulse with BrdU at 37˚C and 70 min incubation with the Anti-BrdU-POD working solution.

Cell density. The cell density at different passages was determined by trypsinizing and counting the viable cells using Trypan Blue solution (0.4%) with a Bürker camera.

Electron microscopy. Cells at different passages, plated in 35 mm petri dishes and allowed to grow reaching the morphology and the degree of confluence as already described above, were fixed 60 minutes at room temperature with 2% glutaraldehyde (GDA) in 0.1 M Sorensen phosphate buffer (pH 7.4), thoroughly rinsed with

the same buffer, post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M Sorensen phosphate buffer, dehydrated through an ascending series of ethanols, and embedded in Durcupan (Fluka Riedel de Haen, Seelze, Germany). Ultrathin sections were obtained with an Ultracut ultramicrotome (Reichert-Jung, Wien, Austria), stained with uranyl acetate and lead citrate before examination using a Jeol CX100 electron microscope (Jeol, Tokyo, Japan).

Statistical analysis. All data were derived from 3 parental cell lines sub cultivated in single flasks for 50 passages, thus the data obtained are expressed as mean values ± standard deviation. Student's *t*-test (independent two population *t*-test performed with Origin 6.0, Northampton, USA) was performed to determine statistically significant differences between the mean values. A p-value of <0.05 was considered statistically significant.

Results

Phase-contrast morphology. Initial morphological differences at different passages of the culture system described above were evaluated by simply observing cell morphology changes. Figure 2A shows cell morphology of Caco-2 cells at passage 6: the cell shapes are not well defined and their contours are broad. In all analyzed fields the cells displayed morphologically heterogeneous features, with rounded cells adjacent to polygonal cells. Figure 2B shows representative example of cells at passage 24: the cell shape is more regular, the cell contours are well evident, but a degree of heterogeneity persists. Figure 2C shows representative cells at passage 41: the cell shape is not very different with respect to cells at passage number 24, but cells appear to be enriched with copious granules. The appearance of granules at this level of cell culture is in agreement with the notion that cells from colon carcinoma, particularly Caco-2 cells, store high quantities of glycogen passing from the exponential phase to the stationary phase of growth (14). A characteristic feature of differentiated Caco-2 cells is the dome formation (Figure 2C, arrow). This specialized structure is found after cell complete differentiation in culture, for instance after 10-15 days of post-confluence (2), or in the presence of particular cell differentiation inducers in the medium, in Caco-2 cells as well as in other cell types (15).

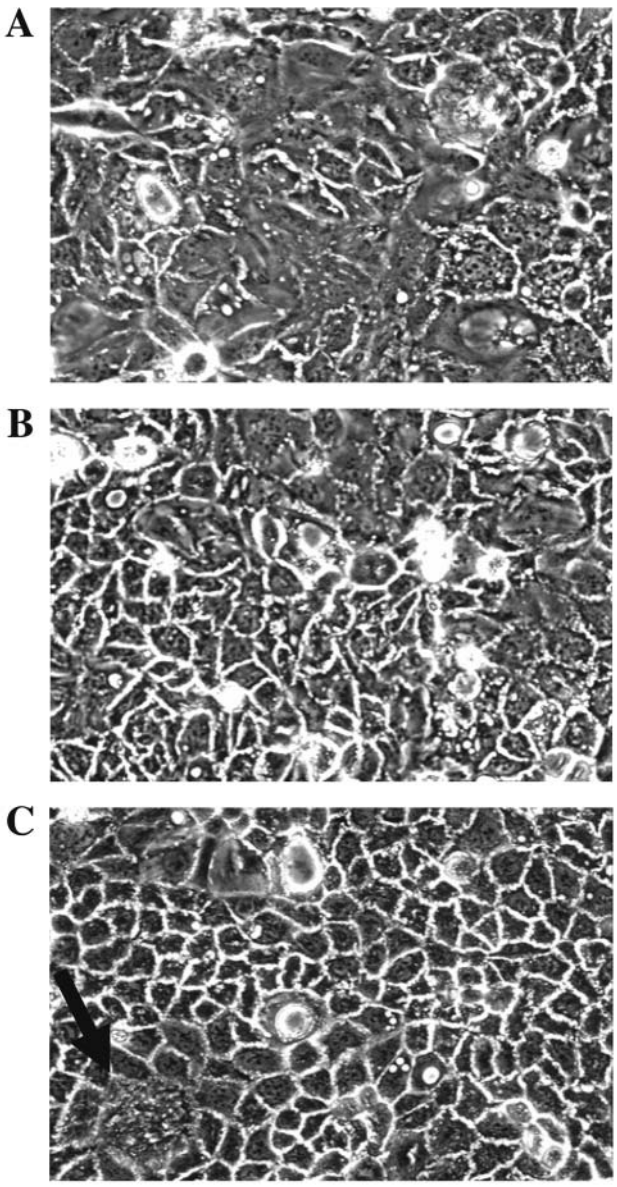

Figure 2. *Phase contrast microscopy of Caco-2 cells at different passage numbers. Cell images of sub-culture at passage 6 (panel A); passage 24 (panel B) and passage 41 (panel C). The arrow indicates the presence of a dome.*

Ultrastructural analysis. Morphological features of non-confluent Caco-2 cells at different passage numbers are given in Table I. Ultrastructural analysis, between passage 4 and 11, showed a monolayer composed mostly of rounded and undifferentiated cells. Thick apical microvilli were scattered (Figure 3A) and lateral junctions were not present (Figure 3B). At later passages cell hypertrophy was detected. A highly organized brush border was harvested from Caco-2 cells composed of elongated and regular microvilli, while desmosomes appeared to be among adjacent cells from passage 18 (Figure 3C). The appearance of tight and adherens junctions occurred at late passages (Figure 3D); concomitantly a well-developed and complete junctional apparatus was present, with the peculiar "kissing points" being localized in the apical zone of the lateral cell membrane. These ultrastructural features were maintained up to passage 48, when Caco-2 cells showed abundant glycogen granules in cytoplasm (data not shown).

Growth assay (proliferation rate and cell density). Figure 4A shows that the proliferation rate of Caco-2 cells diminished progressively with an increasing number of passages during

Table I. *Ultrastructural features of Caco-2 cells at different passages.*

Passage number	4-11	18-25	40-44
Cell monolayer	Rounded cells	Hypertrophic *vs*. 4th-11th passage	Similar to 18th-25th passages
Apical microvilli	±	++	++
Junctional apparatus			
Tight junctions	--	--	++
Adherens junctions	--	--	++
Desmosomes	--	+	++

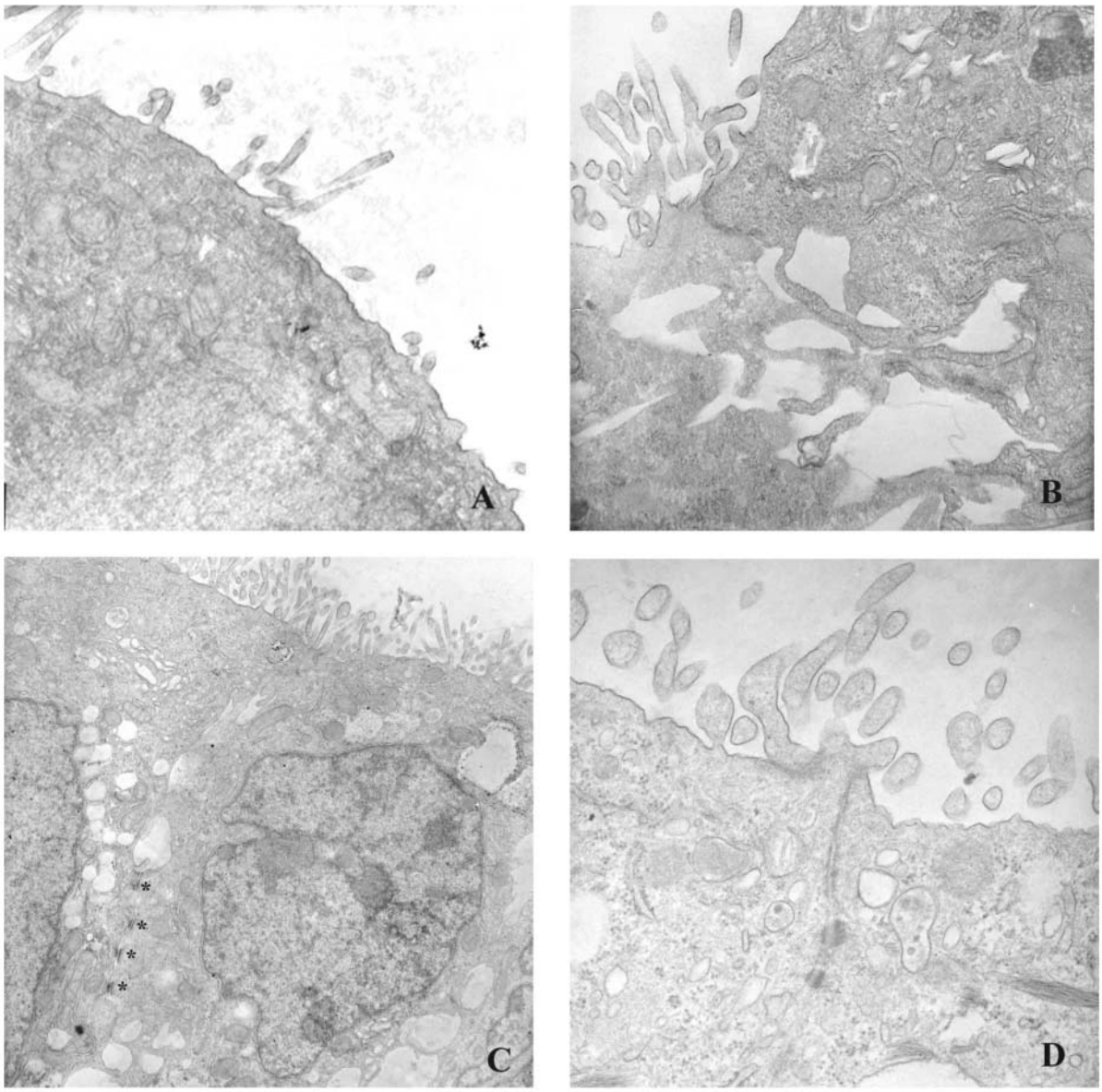

Figure 3. *Transmission electron photomicrographs of Araldite ultrathin sections of Caco-2 cells harvested at different passages. (A) and (B): Passage 11; (C): Passage 18; (D): Passage 40. Asterisks in (C) indicate desmosomes. Original magnification: (A) and (B): x14,000; (C): x4,800; (D): x20,000.*

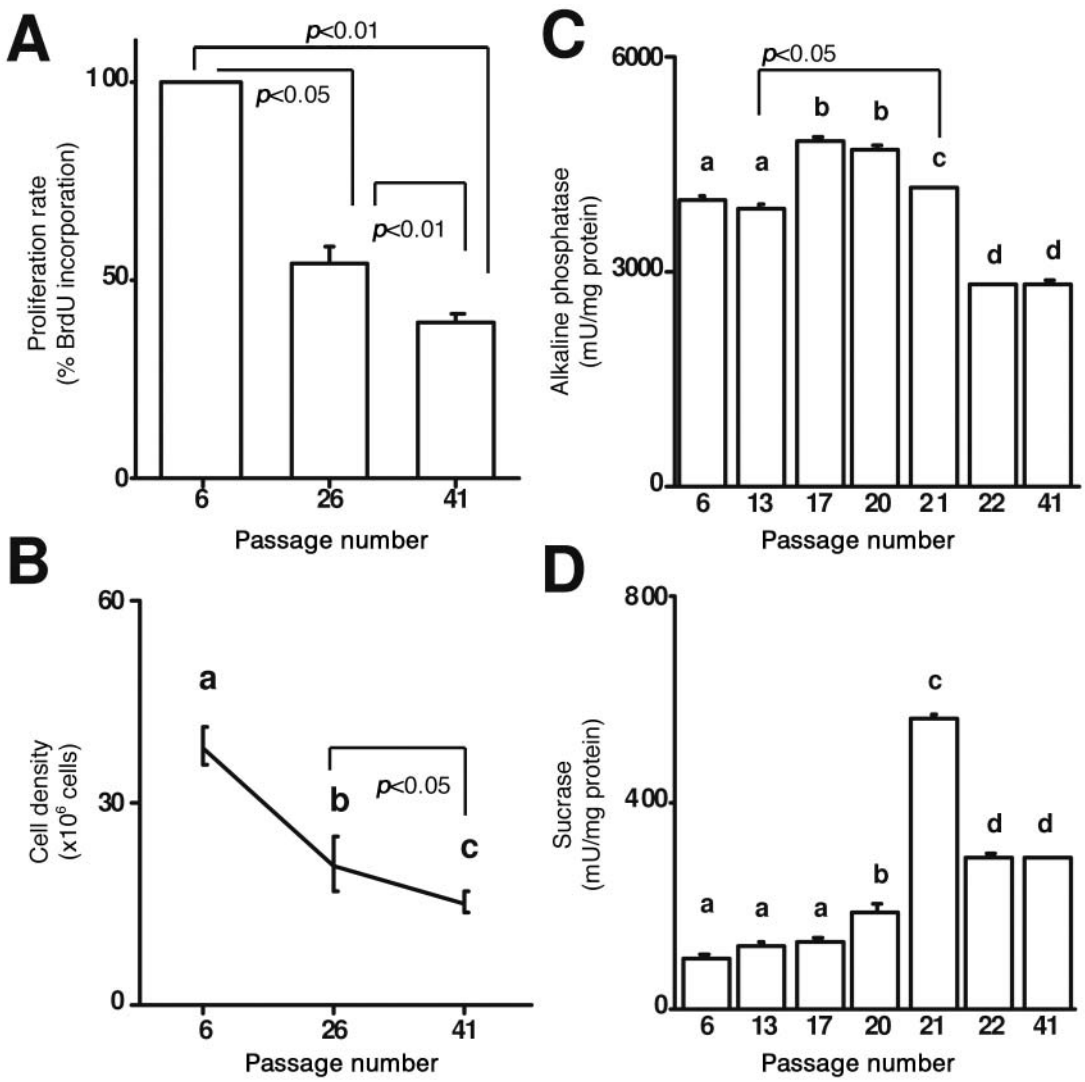

Figure 4. *Effect of passage number on proliferation rate, cell density and enzymatic activities. In panel A the values obtained at passage 26 and 41 were reported as a percentage with respect to the value obtained at passage 6 and evaluated maintaining 80-90% of confluence for all cell passages. Panels C and D show the effect of passage number on alkaline phosphatase (panel C) and sucrase-isomaltase (panel D) activity. Each bar represents the mean ± S.D. of 3 values from 3 batches of cell cultures assayed under the same experimental conditions. Bars with different letters are significantly different at p<0.01.*

the culture periods. These results, together with the cell density calculation (Figure 4B), suggest that Caco-2 cells change their morphology and, presumably, functions under these experimental conditions.

Enzymatic activities. The activities of alkaline phosphatase and sucrase-isomaltase, two well-known biochemical markers of intestinal cell differentiation (2,5), were measured in the brush border fraction (P2) isolated from the cell homogenate. In Figure 4C the alkaline phosphatase activity increased from passage 17 to 21 then decreased reaching a plateau starting from passage 41, an activity trend consistent with previously reported values (16). The sucrase-isomaltase activity, monitored in parallel on the same samples, increased from passage 20, with a maximum at passage 21, to slightly decrease, but remaining always higher than that monitored at the first passages, and reaching a plateau in cells harvested from passage 41 (Figure 4D).

Discussion

The aim of this paper was to report the establishment of a new methodological approach to induce the differentiation phenotype in the Caco-2 cell line. Our initial cell population was a parental cell line, therefore amply heterogeneous with respect to morphology, shape, degree of differentiation (16-

17), and transport rate of glucose and ions (18). Undifferentiated tumor cells are characterized by intense proliferation rate and growth of multiple layers; on the contrary, in cells differentiated as a single monolayer, the proliferation rate is stopped by apoptotic processes activated by contact inhibition. Following the described cell culture conditions, we obtained a well-differentiated population, characterized by distinct stages of differentiation, each of them useful to study not only drug transport and barrier integrity, but also intestinal functions and stages of neoplastic transformation. This result was achieved using standard culture medium, supplemented with fetal calf serum but devoid of substances known to induce cell differentiation such as sodium butyrate (19). Our cell growth conditions are different from others previously based on the use of: a) serum-free media (5, 20), b) serum-free media supplemented with insulin, transferrin and selenium (4), and c) high doses of glutamine (4-6 mM) (6, 21). The main result reported in the present study was the pronounced degree of differentiation in Caco-2 cells prior to their post-confluence, a spontaneous cell differentiation process observed for at least 15-20 days after the stationary stage (2). The growth at post-confluence, although the most utilized and known procedure to differentiate Caco-2 cells, is a situation which although difficult to believe exists *in vivo*, particularly at the intestinal level, where cells undergo a continuous renewal in short times. In fact, the intestinal epithelium is comprised of a tight continuous monolayer both in the crypts, where undifferentiated proliferating cells reside (22) and on the villi, where differentiated cells are present. Phase-contrast morphology, proliferation rate and cell density, strictly related to cell viability, were considered and analyzed to monitor the differentiation process of our parental Caco-2 cell line grown in culture for 50 passages, together with ultrastructure analysis and enzyme activities. All these parameters were constantly evaluated in our cell cultures at 80-90% of confluence and for each parameter we found morphological and biochemical differences between different cell passages. The cellular differentiated phenotype was reached under our protocol through three main steps of sub culturing cells, corresponding to three cell differentiation degrees (Table I and Figure 4): the first stage, from initial passages up to passage number 17, is representative of an undifferentiated phenotype of cells, with low enzyme activities; the intermediate stage, from passage 18 up to 25, is representative of a differentiated phenotype, characterized above all by the presence of a well-developed brush border with microvilli and with high enzyme activities; the third stage, from passage 26 up to 50, is the final differentiated stage of cells, since it is characterized by the presence of microvilli together with the appearance of a well-developed junctional apparatus, confirming the polarity and the transport functions of the cell monolayer, and with plateau values of enzyme activities. On this basis, our cell differentiation procedure offers the possibility to choose, from the same cell population, the differentiation degree of interest simply from a cell passage number. These cells may be frozen at any different degree of differentiation, that is to say, at the different passage numbers; when thawed they retain all the characteristics acquired in original culture before freezing. Several authors analyzed Caco-2 cell differentiation at different passage numbers (17), but in all these previous works the cells were clones of parental cells at different passages and were cultured at post-confluence; in some cases they were also plated on permeable filter supports (8) or on pre-coated plastic area (23).

In summary, the current findings show that a stringent standardization of culture conditions significantly affects the biological and functional characteristics of the Caco-2 cell monolayer, with the possibility of reaching a differentiation degree consisting of cells with characteristic of heterogeneity very close to that observed in the intestinal epithelium (24). Our experimental conditions can be adopted not only in the study of fluorescent drug carriers and microscopy, where post-confluence and/or growth on filter membranes can limit the use of these cell lines, but could also be useful to establish a model of intestinal epithelia for stages in the development and progression of the cancer phenotype.

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