The differentiation process of intestinal epithelial cells is associated with the appearance of statin, a non-proliferation-specific nuclear protein

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Summary

The expression of statin, a 57 000 Mr nuclear protein specifically present in non-proliferating cultured fibroblasts, was studied in vivo in the differentiating epithelial cells of the rat intestine. Using immunofluorescence microscopy we found that undifferentiated, proliferating crypt enterocytes are statin negative, whereas the differentiated non-proliferating villus enterocytes are statin positive. The epithelial cells of the intestine were isolated according to different stages of differentiation and the expression of statin was studied biochemically by immunoblotting assays. The prominent band (57 000 Mr) was present in abundance in villus cell fractions but undetectable in crypt cell fractions. These findings were also confirmed by immunofluorescence microscopy on individual intestinal epithelial cells of the different isolated fractions. The results presented here, which are similar to observations made in cell cultures, suggest that statin is a unique protein associated with the non-proliferative state of differentiated cells in tissue.

Key words: nuclear protein, cell division, intestine.

Introduction

Statin is a unique 57K (K=10^3 Mr) nuclear protein that was first described in quiescent and senescent human cultured fibroblasts (Wang, 1985a). Using light-microscopic and electron-microscopic immunocytochemistry, the protein was shown to be localized at the nuclear periphery (Wang, 1985b). Recent experiments involving subfractionation of rat liver nuclei showed that statin is predominantly present in the nuclear envelope (Wang, 1989). Statin is only expressed, in vitro, in cells that are not proliferating. When proliferating human skin fibroblasts are rendered quiescent by growing them to confluence state or by serum starvation, they start to express statin (Wang, 1987). When serum-starved cells are stimulated to enter the cell cycle by serum addition, the presence of statin becomes rapidly undetectable (Wang and Lin, 1986).

The dynamic correlation between the cessation of cell proliferation associated with differentiation and statin expression observed in cultured cells, has never been well demonstrated in vivo; although statin is known to be present in many different tissues composed of non-dividing cells (Sester et al. 1989; Wang and Krueger, 1985; Muggleton-Harris and Wang, 1989). Using the differentiation of intestinal epithelial cells as a model, in this paper we describe an in vivo system in which the physiological loss of cell proliferation is concomitantly associated with the gradual appearance of statin.

Materials and methods

Immunofluorescence microscopy

Long-Evans male rats (Charles-River Canada) aged between 6 and 8 weeks were used throughout this study. Rats were fed ad libitum and killed by suffocation in a CO₂ chamber. One centimeter of the second portion of the duodenum and 1 cm of the jejunum were excised and cut into small pieces, followed by embedding in O.C.T. Compound (Miles Lab., Indiana) and freezing in liquid nitrogen. Cross-sections of 6 μm were cut with a cryostat and left on a glass slide at room temperature for 2 h before a 10 min fixation in pure acetone at 4°C. The slides were then rinsed in phosphate-buffered saline (PBS) for 5 min and incubated overnight at room temperature with the anti-statin monoclonal antibody (S-44) (Sester et al. 1989) diluted 1:1000 in PBS. Next morning the slides were rinsed again in PBS (3x5 min), incubated with a goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (Cappel-Organon Teknika, Scarborough Canada) for 30 min, rinsed again in PBS (3x20 min) and mounted in medium containing 50% glycerol–50% PBS. For each slide that was incubated with S-44 a parallel control was done in which a slide was incubated with a nonspecific monoclonal antibody (PA1 ascites) instead of S-44. The sections were examined with a Nikon fluorescence Labophot photomicroscope equipped with epi-illumination.
Intestinal epithelial cell isolation and fractionation

The first half of the small intestine was recovered (from the beginning of the duodenum to the middle of the jejunum) and intestinal epithelial cells, located from the top of the villus to the bottom of the crypt, were isolated using Weiser's (1973) method. Briefly, the lumen of the intestine is washed with saline and is filled with the first solution (solution A), which contains 27 mM-sodium citrate, 1.5 mM-KCl, 96 mM-NaCl, 8 mM-KH₂PO₄, 5.6 mM-Na₂HPO₄ at pH 7.3. After 15 min, solution A is discarded and the lumen is then filled with the second solution (solution B) containing PBS, 1.5 mM-EDTA and 0.5 mM-Dithiothreitol. This solution selectively releases epithelial intestinal cells from their basement membrane; the cells at the top of the villus are the first to come off. At different times solution B, containing the epithelial cells, is removed from the intestinal lumen and kept on ice. After each removal, new solution B is added to the lumen and removed after additional epithelial cells are released from the lining of the intestine.

Solution B, in the lumen, is thus changed eight times to give 8 intestinal fractions (see Table 1). The quality of the cell isolation was monitored by measuring the alkaline phosphatase activity for each fraction. Additional assessment of the cell isolation was done by examining morphologically cell samples from each fraction under phase-contrast optics.

For immunofluorescence microscopy with statin antibody (S-44), isolated epithelial cells from villus (fraction 1) and crypt (fraction 8) regions were suspended in phosphate-buffered saline (PBS). Portions of these cell isolates were put on glass slides and left to dry in air at room temperature. These cell smears were then fixed by incubation in 50% acetone/50% methanol for 10 min at −20 °C. The specimens fixed on the glass slides were then rinsed in PBS and processed for staining with anti-statin antibody as previously described for the whole tissue.

Western blotting for protein identification

Approximately 500 µl of packed isolated intestinal cells from each of the eight fractions (crypt and villus fractions) was mixed with 500 µl of 20 mM-Tris−HCl buffer (pH 7.5), containing 1 mM-phenylmethylsulphonyl fluoride (PMSF), homogenized using a Potter homogenizer (15–20 strokes) and sonicated for 3 min. To all homogenates, a solution of 20 mM-Tris−HCl (pH 7.5) containing 1 mM-PMSF was added to make a total volume of 5 ml. All fractions were centrifuged for 10 min at 3000 revs min⁻¹ to obtain an insoluble nuclear fraction containing a portion of the cytoskeleton. The pellet was collected and resuspended in 5 ml of the Tris solution. Samples of this solution were taken for each of the eight fractions to determine the protein concentration using the Bio-Rad protein assay. For each fraction a total of 120 µg of protein was loaded in each lane and polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to Laemmli's (1970) technique. The proteins on the SDS−PAGE gel were transferred to nitrocellulose paper (Schleicher and Schuell, Keene, NH) by electrophoretic for 4 h at 325 mA (Towbin et al. 1979). The blots were blocked with Tween-20 in Tris-buffered saline (TBS; 20 mM-Tris−base, 500 mM-NaCl at pH 7.5) for 1 h, incubated with the statin antibody (S-44) overnight, blocked again with Tween-20 in TBS, incubated with a secondary rabbit anti-mouse antibody. Following an additional step of blocking, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody according to a previously published procedure (Sester et al. 1989). A control was also performed in which the statin antibody (primary antibody) was replaced with PBS.

Double labelling of autoradiography for [³H]thymidine incorporation and immunofluorescence microscopy

Three-month-old rats were injected intraperitoneally with 300 µCi of [³H]thymidine and killed 15 min later. The duodenum was removed and cut into cryostat frozen sections of 10 µm and incubated with anti-statin antibody for immunofluorescence microscopy. After photography of the designated area, the section was covered with radioautographic emulsion and exposed for 11 days. The detailed procedure for processing has been described (Cheng and Leblond, 1975). The same designated areas were photographed again with phase-contrast optics for the radiographic labelling of [³H]thymidine. The position of antibody staining and the autoradiography can then be correlated by comparing the presence of the nuclear ring structure by fluorescence optics and grain labelling by phase-contrast optics.

Results

Immunofluorescence microscopy

Identification of statin was performed on frozen sections of intestine (Fig. 1A–D). Undifferentiated dividing epithelial cells located at the base of the crypt were not stained with the anti-statin antibody (Fig. 1C). From the middle of the crypt to the top of the crypt weak nuclear ring staining was observed in epithelial cells; the crypt cells located in the basal area were not labelled. However, the statin staining was heterogeneous in the upper half of the crypt, some cells appeared labelled whereas adjacent ones appeared unlabelled (as opposed to the villus where all the epithelial cells appeared to be homogeneously labelled). At the crypt–villus junction there is an increase in nuclear ring staining intensity in intestinal epithelial cells (Fig. 1C); villus cells were all uniformly labelled with the statin antibody S-44 (Fig. 1B). At the top of the villus (Fig. 1A), epithelial cells still exhibit nuclear ring staining, although the staining is again heterogeneous, with some cells being strongly positive while adjacent ones showed only weak statin staining. It was also observed that at the villus top the nuclear ring staining was sometimes irregular in shape, and sometimes smaller than in the main body of the villus.

Intensely stained flat cells were seen apposed to the crypts throughout the intestine; these cells are believed to

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<th>Isolated intestinal cell fraction number</th>
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The intestine was filled with a sample of solution B (see Materials and methods) and after each time interval the solution (containing the epithelial cells) was recovered and the cells were rinsed in PBS. Each sample of solution B consisted in an isolated intestinal cell fraction. Fraction 1 being villus tip cells and fraction 8 crypt cells. The method described here is a modification of the previously published procedure of Weiser (1973).

Table 1. Method of intestinal cell isolation
be peri-cryptal fibroblasts (Fig. 1C). Also strongly stained cells of different sizes and shapes were seen in the core of the villus, these cells are a mixture different cell types like fibroblasts and endothelial cells, which are presumably statin positive, since most of them are in the quiescent state. Sections of the intestine stained with a control monoclonal antibody were seen to be devoid of staining (Fig. 1D). In addition, myocytes of the muscle layers exhibited strong nuclear ring staining (Fig. 2) and the two layers, circular and longitudinal muscle, can easily be distinguished by the horizontal and longitudinal orientation of the statin-positive nuclei.

**Characterization of isolated intestinal epithelial cells**

The quality of the intestinal crypt and villus cell isolation procedure was assessed by both an alkaline phosphatase enzyme assay and by morphology. The activity of alkaline phosphatase (data not shown) was very high in the first (villus cells) fractions; it then decreased to reach a minimum in fraction 9 (crypt cells). This is consistent with the known abundance of that enzyme in the differentiated villus cells as opposed to their undifferentiated crypt counterpart (Weiser, 1973). Morphologically, the villus tip fraction showed numerous large flat single cells (Fig. 3B) with a polarized region of dense microvilli. In addition the presence of aggregates of round cells with a general morphology compatible with villus tips was noted (Fig. 3A). On the other hand the crypt base fraction showed the presence of small spindle-shaped cells (Fig. 3D) with shorter and sparse microvilli, the aggregated form of those cells shows a conical 'crypt-like' morphology (Fig. 3C).

The method used in this study to isolate intestinal epithelial cells allows us to establish a differentiation gradient among the eight isolated intestinal fractions: fraction 1 of villus tip cells being the most differentiated, and fraction 8 of the dividing crypt cells being the most undifferentiated cells.

Biochemical characterization of the presence of statin was performed by the immunoblotting technique on the different isolated intestinal cell fractions with the anti-statin monoclonal antibody (S-44). Fig. 4 shows that statin is abundant in the villus intestinal cells (fractions 1–4) and its presence gradually decreases to an undetectable level in the crypt epithelial cells (fraction 8).

Two bands can be seen in intestinal cells after immuno-
Fig. 2. Expression of statin in the muscle layers of the duodenum as revealed with the anti-statin antibody (S-44). The nuclei of muscle cells of both the circular muscle layer (cm) and the longitudinal muscle layer (lm) show intense nuclear ring staining with the statin antibody. The epithelial cells located at the bottom of the crypts are statin negative (arrowheads).

Fig. 3. Phase-contrast microscopy of isolated villus and crypt cells. A. Aggregate of villus cells after isolation from intestine; the general morphology of the aggregate resembles a villus tip. B. Isolated villus cell with dense microvilli layer (arrowheads). C. Aggregate of crypt cells after isolation from intestine; the general morphology of the aggregate resembles the bottom of a bifide crypt. D. Isolated crypt cell with shorter and scattered microvilli (arrowheads).
Kim and Lin (1986) demonstrated that when serum-starved quiescent cells are stimulated to proliferate by raising the serum concentration of the medium from 0.5% to 10%, statin rapidly becomes undetectable (Fig. 5A and B). This disappearance of statin occurs before the onset of DNA synthesis (Fig. 5C). Isolated epithelial cells of the villus are only weakly stained or not stained at all after incubation with the anti-statin antibody (Fig. 5D and E); the incubation with the control monoclonal antibody is also negative (Fig. 5F).

Comparison of statin-negativity and DNA synthesis positivity in the crypt cells is shown in Fig. 6. The duodenal section of the rat intestinal tissue is processed for immunofluorescence microscopic staining for the presence of statin and autoradiographic labelling for DNA synthesis activity. As shown in Fig. 6, comparison of fluorescence (A) and phase-contrast images of the same area in the villus shows that the statin-positive cells in the villus tip do not have DNA synthesis activity, as indicated by the absence of autoradiographic grains. In contrast, the cells in the crypt region are negative for statin as shown by the absence of nuclear ring staining; these cells show the presence of abundant grains, indicating DNA synthesis activity. Here a small fraction of the statin-negative cells are not labelled with autoradiographic grains, either because the cells are in a cell cycle phase other than the S-phase or because their DNA synthesis activity is too low to be detected by the procedure employed here. Nevertheless, the statin-negative cells of the crypt region in general are positive for DNA synthesis.

**Discussion**

In fibroblasts, the nuclear protein statin is expressed only in quiescent cells. When serum-starved quiescent cells are stimulated to proliferate by raising the serum concentration of the medium from 0.5% to 10%, statin rapidly becomes undetectable (Fig. 5A and B). This disappearance of statin occurs before the onset of DNA synthesis (Fig. 5C). If these actively dividing young fibroblasts are grown to confluence, they stop dividing, stop synthesizing DNA and start to express statin (Fig. 5D and E). The present report was designed to examine the expression of statin in a well-defined in vivo tissue system, where the cessation of proliferation is coupled in a coordinated fashion with the differentiation process. For this purpose, the epithelial cells lining the intestinal lumen were chosen as a model for our study.

The intestinal epithelial system is ideal for such a study, because there is a compartment of young, undifferentiated dividing cells; these cells actively synthesize DNA in the crypt (Cheng and Leblond, 1974). The crypt cells divide and migrate up along the crypt to give rise to villus cells, which are more differentiated, non-dividing cells. These cells, although very active in RNA and DNA synthesis,
protein synthesis (Uddin et al. 1984), do not synthesize DNA. Differentiated cells migrate along the villus where they exert their function as the absorptive intestinal cells. When their function is completed and the cells become terminally differentiated they are extruded into the lumen of the intestine at the tip of the villus. Although it has been known for a long time that intestinal epithelial cells stop dividing when they move from the crypt to the villus, the underlying mechanism that is responsible for this phenomenon is still unknown. Recently, transforming growth factor \( \beta \) (TGF-\( \beta \)) has been shown to be associated with villus cells and to inhibit proliferation of a crypt cell line in vitro (Barnard et al. 1989). In addition, recent work with transgenic mice using the 5' untranslated regions of the liver and intestinal fatty acid binding proteins (L-FABP and I-FABP) has attempted to unravel the molecular mechanism of differential gene expression along the crypt-villus axis (for review, see Gordon, 1989).

In this study we have shown that the cessation of proliferation observed when crypt cells migrate to the villus is closely associated with a gradual appearance of statin in the nucleus. Although we did not study individual cells, the general trend from crypt base to crypt top is that the actively dividing crypt cells are statin-negative, when they move upwards in the crypt and cease to proliferate they gradually start to express a positive nuclear ring staining with the anti-statin antibody in immunofluorescence microscopy; they stay statin-positive until their extrusion into the lumen, at the tip of the villus. The pattern of statin expression observed here is thus very similar to the one displayed by the in vitro cultured fibroblasts. Therefore, the presence of statin can be used as a marker for differentiated intestinal epithelial cells. Availability of such a marker paves the way for unravelling the mechanisms responsible for the differentiation process in these cells.

The molecular weight of statin (major band of 57K) as determined by immunoblotting assays in the intestine, is the same as observed in cultured fibroblasts (Wang, 1985a) and in rat liver (Sester et al. 1989). The presence of a lower molecular weight band, after immunoblotting with the anti-statin antibody (S-44), was not observed in any of the eight fractions in a control blot where PBS was used instead of S-44 (not shown), which suggests that the band is not produced by direct binding of the secondary or the tertiary antibody. The 50K band could well result from a cross-reaction of the anti-statin antibody with another protein; however, it is interesting to note that the 50K band decreases, like the prominent 57K band, from fraction 1 to 8; we thus cannot exclude the possibility that the lower \( M_r \) band might be a degradation product of the 57K band.

Finally, the similarities between the cultured human fibroblasts and the rat intestinal epithelium in their expression of statin in nonproliferating cells, in the molecular weight of statin and in their statin nuclear ring immunofluorescence staining, suggest that statin may be
Fig. 6. Colocalization of statin and DNA synthesis in duodenal tissues. The detailed procedure for processing the section for fluorescence antibody staining and autoradiography is described in Materials and methods. A. Villus cell nuclei all express statin as seen by their strong nuclear ring staining (arrowheads) after immunofluorescence microscopy. B. The same area of A viewed by phase-contrast optics showing absence of autoradiographic grains (arrowheads). C. Crypt cell nuclei are negative for statin under fluorescence optics (arrows). D. Same area of C showing the activity of DNA synthesis by the presence of grains. ×450.

a conserved nuclear protein universally associated with the non-proliferating state, both in cell culture and in vivo.

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Statin expression in intestinal cells
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