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Morphology and Intermediate Filament Composition of Human Mammary Epithelial Cells Treated with Stable Butyrate Derivative

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Abstract. A new stable butyrate derivative monobut-3 was previously shown to inhibit proliferation and promote differentiation in human mammary established cell lines. The present study on monobut-3's effects on mammary epithelial cells cultured from human non-malignant and malignant breast tissues demonstrated pronounced morphological alterations suggestive of cellular differentiation. In addition, some degree of architectural differentiation was also evident in treated primary cultures. Monobut-3 did not affect the expression of vimentin and cytokeratin 18 when assessed in human breast cell lines expressing one or both types of intermediate filaments. However, it did induce expression of cytokeratin 19, characteristic of fully differentiated mammary cells, in one of the two cell lines devoid of this cytokeratin subtype. Furthermore, the network of intermediate filaments was often more largely extended in cells treated with monobut-3 than in untreated ones. These results indicate that monobut-3 can induce subtle changes in intermediate filaments which may contribute to its ability to promote differentiation in human mammary cells.

Sodium butyrate has been shown to be a potent cytostatic and cytodifferentiating agent for a large number of tumor-derived cell lines in vitro (1-3). However, clinical trials of n-butyrate reveal a low potency probably due to the rapid metabolism and high clearance rate of these compounds (4,5). Recently, several laboratories have developed pro-drug derivatives by covalently linking the butyric acid molecule to polyhydroxylated carriers. These derivatives appear more stable in vivo and potentially more efficient as antitumor agents (6-10).

We have recently demonstrated that two of the above new derivatives, namely "monobut-3" [3-O-butyloyl-1,2-O-isopropylidene-α-D-glucopyranose], and "monobut-6" [6-O-butyloyl-1,2-O-isopropylidene-α-D-glucopyranose], inhibited the proliferation and modulated the cellular morphology and the expression of cell surface antigens and estrogen receptors in human mammary established cell lines (11). This prompted us to extend our findings to primary cultures of epithelial cells derived from human breast tissues. Pronounced morphological alterations suggestive of cellular differentiation were observed. Morphological changes have long been attributed to alterations in the organization of actin cytoskeleton (12). However, recent data suggest that the intermediate filament cytoskeleton may also be implicated, especially in the morphological changes associated with transformation (13). In addition to cellular morphology, alterations in the composition of intermediate filaments have also been correlated with the neoplastic phenotype in human breast cancer cells (14). Therefore, we were interested to determine whether cellular differentiation induced by butyrate derivatives was also accompanied by a modification of this type of cytoskeletal filaments in human mammary cells.

The present report 1) describes the morphological effects of monobut-3 in primary cultures of human breast tissues from non-malignant and malignant origin and 2) examines the ability of the derivative to modulate the expression and distribution of cytokeratin and vimentin intermediate filaments in human breast cell lines. Established cell lines rather than primary cultures were chosen because they generate a larger number of cells thereby allowing a parallel assessment of both parameters.

Materials and Methods

Materials. Monobut-3 was synthesized as previously described (6). Butyric acid was covalently bound to polyhydroxylated compound in anoxic solvents. The mouse monoclonal antibodies (MAbs) against human cytokeratin (Ck) 18 (RPN1100), Ck 19 (RPN1165) and human vimentin (RPN1102) were purchased from Amersham (Les Ulis, France). Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (IgG-FITC) were also purchased from Amersham.

Cell cultures. The 4 primary cultures of human mammary epithelial cells studied were obtained from breast biopsies performed in 1) non-
Figure 1. Morphological effect of monobut-3 on primary cultures of mammary epithelial cells from normal tissue adjacent to breast carcinoma (A, B), non proliferative benign tumor (C, D), ductal (E, F) and lobular (G, H) carcinomas. Phase-contrast micrographs represent controls (A, C, E and G) and cells treated with 4mM of monobut-3 for 6 days (B, D, E and F). Scale bars = 25μm.
malignant tissue adjacent to invasive carcinoma; 2) non-proliferative benign tumor; 3) ductal carcinoma of grade I; 4) lobular carcinoma. Cells were isolated and cultured in the PC medium developed for growing epithelial cell as previously described (15).

The human breast tumor cell lines, MCF-7 and MDAMB-231, derived from metastatic pleural effusions and were kindly provided by Dr F. Calvo (Hôpital St Louis, Paris). The spontaneously immortalized normal cell line HBL 100 provided by Dr R. Cassingena (IRSC, Villejuif) derived from the milk of an apparently healthy woman but contained SV40 genetic information (16). The two new immortalized non-malignant cell lines, NPM14-T and NBAT32 T, were established and characterized in our laboratory (17,18). They derived from a non-proliferative benign tumor and from a non-malignant tissue adjacent to an invasive breast carcinoma, respectively. Cells were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum (FCS) and antibiotics, in a 5% CO2 humidified atmosphere at 37°C. All cell lines were routinely passaged once a week at a 1:10 split ratio.

Cellular morphology. Mammary epithelial cells from confluent primary cultures were trypsinized and seeded into 25-cm2 Costar flasks at a 1:2 split ratio in PC medium. Monobut-3 was added to the culture medium at a concentration of 4 mM for 6 days. Morphological alterations were assessed and compared to control cells under phase-contrast microscopy.

Analysis of cytokeratin and vimentin intermediate filaments. For these studies, exponentially growing cells from the various cell lines were treated with 4 mM of monobut-3 for 3 days. Cytokeratin and vimentin expression was assessed by flow cytometry as previously described (11). Briefly, cells in suspension were fixed with 70% ethanol at -20°C for 1 h, washed twice in PBS with 1% FCS, resuspended in 100 μl MAB diluted 1:10 in PBS and incubated at room temperature for 45 min. Cells were then washed twice with PBS, incubated with 100 μl anti-mouse IgG-FITC diluted 1:10 for 45 min at room temperature in the dark. After rinsing with PBS, cell concentration was adjusted to 1 x 10⁶ cells/ml before analysis in a FACSscan Flow cytometer (Becton Dickinson, Grenoble, France).

To examine the distribution of cytokeratin and vimentin within the cells, controls and cells treated with 4 mM monobut-3 were grown for 3 days on 8-chamber Lab-Tek glass slides (Intermed, Paris, France), fixed in cold methanol and prepared as previously described (18). Fluorescence was observed with a Zeiss photomicroscope.

Results

Morphological differentiation induced by monobut-3 in human breast primary cultures. Monobut-3 induced a profound morphological alteration in the 4 primary cultures deriving from breast tissues regardless of whether they were benign or malignant. Although originally different, the cellular morphology appeared very similar in all treated cultures: cells became larger, acquired a granular appearance and contained cytoplasmic lipid-like vacuoles (Figure 1). These overall cellular changes have been previously shown to be associated with the promotion of differentiation in human breast cancer cells (19). With the exception of the lobular carcinoma-derived culture, monobut-3 also induced some degree of architectural organization as evidenced by cells lining empty spaces where occasionally refringent particles were seen (Figure 1). It is noteworthy that monobut-3 failed to induce any cellular rearrangement in human breast established cells.
Figure 3. Effect of monolayer on the distribution of intermediate filaments in human breast cell lines. Fluorescent micrographs of control MDAMB-231 (A) and NB437-7 (B) cells and cells treated with 2% DMSO for 3 days (C and D), respectively. Scale bar = 25 μm.
Expression and distribution of intermediate filaments in human breast established cell lines. Most cells usually express only one type of intermediate filament; indeed, intermediate filament proteins have become a standard marker for identifying the tissue origin of a cell. However, many epithelial cells in culture are found to express both the epithelial (cytokeratin) and mesenchymal (vimentin) intermediate filament types (20). The breast cell lines studied, except MCF-7, also expressed high levels of vimentin as assessed by flow cytometry. No significant alteration in vimentin expression was, however, observed upon treatment of these cells with monobut-3 (data not shown). For cytokeratin expression, monobut-3 did show some activity. In these studies, two MAbs were used which react solely with Ck18 and Ck19, the two predominant cytokeratin subsets reported to be present in fully differentiated mammary epithelial cells (22). As illustrated in Figure 1A, all breast cell lines showed high levels of Ck18 expression which was not affected by monobut-3. By contrast, only 3 of the 5 cell lines investigated (i.e: MCF-7, NPM14-T and NBAT32-T cells) highly expressed Ck19 while 2 others (i.e: MDAMB-231 and HBL100 cells) lacked Ck19 expression. In the former, monobut-3 had no effect on Ck19 expression while differential effects were detected in the latter. Thus, monobut-3 restored to some extent the expression of Ck19 in HBL100 (Figure 2D) but not MDAMB-231 cells (Figure 2B).

When visualized by immunofluorescent staining, the pattern of vimentin and cytokeratin distribution within the cells expressing one or both types of proteins appeared similar in all cell lines before monobut-3 treatment. Thus vimentin and cytokeratin were distributed around the nucleus with few filaments extending into the cytoplasm (Figure 3 A and C). In addition, a brightly stained juxtanuclear condensation of cytokeratin filaments was also seen in many cells. After treatment with monobut-3, most cells exhibited a network of well-defined vimentin and cytokeratin filaments radiating out from the nucleus to the cell periphery (Figure 3 B and D). The degree of intermediate filament extension induced by monobut-3 showed, however, a certain variability depending on the intermediate filament and the cell type analyzed. Cytokeratin filaments were extensively developed in MDAMB-231 while they were modestly extended in the four other cell lines. Opposite effects were found with vimentin filaments which demonstrated a pronounced extension in NBAT32-T, NPM14-T and HBL100 cells while being barely affected in MDAMB-231 cells.

Discussion

This study shows that the new stable butyrate derivative monobut-3 induced a pronounced morphological differentiation in primary cultures of human mammary epithelial cells derived from both non-malignant and malignant breast tissues. This effect of monobut-3 was characterized by marked cell enlargement, apposition of cytoplasmic granulations and lipid-like vacuoles suggestive of secretory functions. Concomitant with the induction of morphological alteration, monobut-3 restored some spatial cell arrangement reminiscent of alveolar structures in the mammary gland. The induction of morphological and architectural differentiation appeared more pronounced and more consistent in primary cultures than in established mammary cell lines. In the latter, monobut-3 had no or minimal effects on cell enlargement, did increase cell granularity but at variable levels according to the cell line and failed to induce any cellular rearrangement (11). It thus seems that cells in primary cultures retained a greater potential capacity for differentiation than did cells in permanent cell lines.

The implication of intermediate filaments in the phenotypic changes associated with malignant transformation remains unclear. Sommers et al (14) have suggested that coexpression of vimentin with cytokeratins might be a typical feature of highly malignant hormone-independent breast carcinoma cells. However, our own results on a large number of primary cultures (21) from normal, non-malignant and malignant breast tissues showed that vimentin was consistently expressed, together with high levels of cytokeratins, in most epithelial cells regardless of their tissue source. Expression of vimentin did not appear to correlate with malignant properties either. As shown in this study, the four breast cell lines MDAMB-231, NBAT-32T, NPM14-T and HBL100 which were devoid of estrogenic receptors (11,15) did express vimentin even though only one, MDAMB-231, was malignant. These results, together with those of others (20), suggest that vimentin expression is the rule rather than the exception in cultured epithelial cells. This may be due to in vitro growth conditions and perhaps to selection for a more rapidly proliferating dedifferentiated epithelial cell type. Similarly, the differences seen in the expression of breast tissue-specific cytokeratin subsets between cell lines may also be attributed to adaptation to growth in vitro. As reported here, expression of Ck 19, a characteristic of differentiated luminal mammary cells (22) and breast carcinoma-derived cell lines, was found to be retained in three cell lines, but lost in two others.

Treatment with monobut-3 did not produce dramatic alterations in the expression of vimentin and cytokeratins. Indeed, the only detectable effect was a slight induction of Ck19 in non-malignant HBL100 cells, one of the two cell lines which demonstrated a lack of expression before treatment. In all other cell lines, the levels of expression of vimentin and Cks18 and 19 remained unchanged. However, significant modifications in the pattern of arrangement of intermediate filaments within the cells could be detected. While vimentin and cytokeratin filaments were mostly concentrated around the nucleus in non-treated cells, a marked extension of intermediate filaments throughout the cytoplasm was
observed when the cells were treated with monobut-3. Curiously, the most pronounced changes in intermediate filament distribution were observed in the cell lines which showed the lowest responses to monobut-3 in terms of inhibition of cellular proliferation and induction of morphological changes (11). This may indicate that these differentiation-related parameters within the same cell type behave independently of each other under monobut-3 treatment. Alternatively, the maximal level for intermediate filaments to extend may vary according to the cell type. Important intermediate filament rearrangement which resembled the fribular pattern of normal parenchymal cells has been reported in cultured hepatoma cells treated with butyrate (23). Although monobut-3 did not produce strong changes in the composition and distribution of intermediate filaments in human mammary cell lines, it remains possible that the subtle alterations described may be part of the cellular processes contributing to the differentiating effects of this compound.

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