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Application of high content biology demonstrates differential responses of keratin acetylation sites to short chain fatty acids and to mitosis

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ABSTRACT

The intermediate filament cytoskeleton in epithelial tissues is formed of keratin heterodimers. Keratins are highly post-translationally modified proteins, with tyrosine phopsphorylation, serine phosphorylation, and glycosylation amongst reported modifications. We and others have recently reported multiple acetylation sites on keratin 8 and we have previously shown that these sites are responsive to butyrate. In this study, we report the application of cellomic approaches to demonstrate differential responses of three lysine acetylations (lys 10, lys 471 and lys 482) to different short-chain fatty acids. The data imply no fixed hierarchy of acetylation on keratin 8, and furthermore imply different ranges of histone deacetylase (HDAC) inhibitory specificities for short chain fatty acids (SCFA). Furthermore we have used the functionality of the High Content Analysis (HCA) platform to show that the acetylation sites are differentially modified in cells undergoing mitosis. Taken together the data imply distinct roles for keratin acetylations in function.

Keywords: Keratin; Acetylation; Colorectal cancer; Short chain fatty acids; High content biology.

1. Introduction

Short-chain fatty acids (SCFAs) are the principal by-products of fibre fermentation in the gastrointestinal epithelium. The majority of SCFAs comprise of acetate, propionate, and butyrate with contributions of valerate and branched chain fatty acids (BCFA). All SCFAs are weak acids existing predominantly in the anionic, dissociated form in the colonic lumen(1). Butyrate has been shown to induce apoptosis (2), inhibit proliferation (3, 4), and promote a more differentiated phenotype (3, 4). This is considered to be through its ability to regulate gene expression by inhibition of the histone deacetylases (HDAC) (5, 6), which also regulate many non-histone proteins including transcription factors such as Sp1 (7), nuclear structural proteins (8), and p53 (9). We have recently shown keratin 8 (K8), an intermediate filament (IF) protein of the colonocyte cytoskeleton, is acetylated and its acetylation is butyrate-responsive (10).

Intermediate filaments (IFs) are one component of the eukaryotic cytoskeleton. They are formed from heterodimers of type 1 and type 2 keratins. Among various keratins present in colonocytes, keratin 8 and 18 (K8, K18) is the principal pair (11). Other keratins include K7, K19, and K20. Dimerisation of keratins is mediated through pairing along their coiled-coil domains and is regulated, at least in part, through post-translational modification of the globular domains outside these regions. Keratins provide mechanical strength to epithelia in general and enable colonocytes to resist various chemical and mechanical stresses. They are also involved in various regulatory functions of cell including cell cycle, cell differentiation and apoptosis (12). Importance of K8 in intestinal epithelium was demonstrated through generation of K8 knockout mice. K8 null mice reaching to adulthood showed high rates of gastrointestinal epithelial inflammation and colonic hyperplasia (13) and also showed impaired electrolyte and fluid transport (14). In humans a subset of patients with inflammatory bowel disease (ulcerative colitis) has been shown to carry missense mutations in the Keratin 8/18 genes. Reconstructions of these mutations *in vitro* (K8: G62C, I63V, K464N; K18: S230T)

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were associated with reduced filament assembly (15). An important characteristic of keratins is their relative stability of expression even after transformation to pathological state including transformation of normal cells into malignant cells. This property has enabled keratins to be applied as tumour markers (16). Changes in K8 expression during colorectal adenomagenesis and carcinogenesis have been studied by Polley et al (17) by comparing protein expressions in normal and tumour (adenoma, carcinoma) tissue. They found the up regulation of various isoforms of K8 in morphologically normal mucosa from polyp and cancer patients compared with normal mucosa of patients with no pathology. Also, four K8 isoforms were over expressed in polyp mucosa relative to normal mucosa and seven K8 isoforms were over expressed in cancer mucosa relative to healthy mucosa. These findings indicate that expression or modification of K8 alters as the adenoma-carcinoma sequence progresses. Another study showing differential K8 expression in colorectal carcinoma has shown reduced expression of K8 in colorectal cancer is significantly associated with shorter patient's survival (18).

The structural and regulatory functions of keratins are in turn regulated, at least in part, by various post translational modifications (PTMs). Indeed K8 is reported as a highly post -translationally modified protein (Fig 1). The majority of PTMs occur in the globular N- and C-terminal domains, although several occur in the coiled-coil domain (Fig 1). The PTMs of keratins include: phosphorylation, glycosylation and transglutamination which occur on either C or N-termini. These modifications have many suggested implications on the filament assembly and solubility (19). K8 phosphorylation has been shown to regulate filament formation, cell signalling, and protein-protein binding (20) in response to cellular stress, mitosis, and apoptosis (21, 22). We have reported K8 and K18 as highly acetylated (10) and associated alterations in acetylation with depolymerisation (23). Further acetylations have been reported for K8 (24) , and K8 now features 11 empirically determined acetylation sites. We have shown that butyrate causes an K8 acetylation using a high-content biology approach (23).

High-content biology (HCB, also known as High-Content Analysis (HCA), sometimes termed cellomics) is a high-throughput microscopy approach where multiple endpoints may be analysed in both a qualitative and quantitative way integrating the functionalities of both microscopy and flow cytometry (25). HCB is increasingly used in the pharmacology sector for analysis of multiple endpoints in screening cellular responses to compound libraries (26).

Although cellular responses to butyrate have been widely studied, the response to other SCFAs which also occur in the colon lumen at pharmacologically relevant levels is less explored. The HCB approach to analysis of keratin acetylation previously reported (23) was here applied to the analysis of response to other SCFAs.

2. Material and Methods

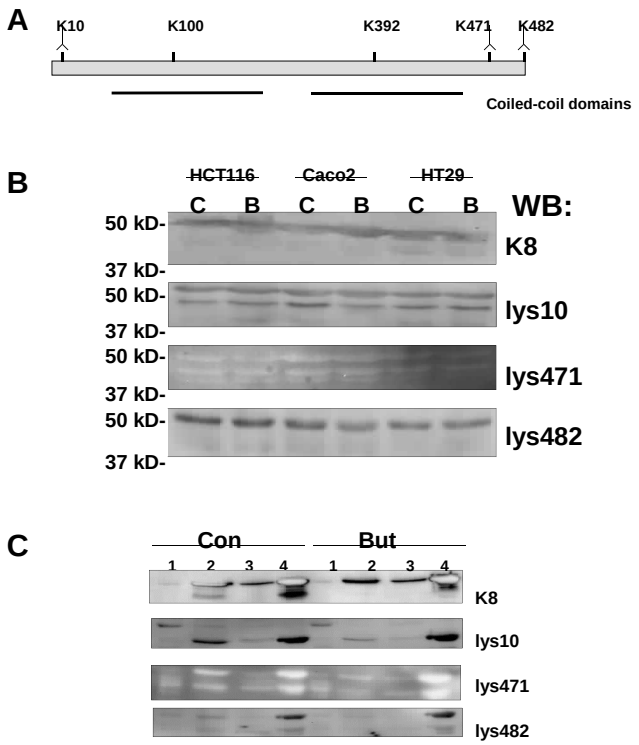

Figure 1.*Different isoforms of K8 are acetylated at different positions.* Panel-A shows the five positions identified to be acetylated. Two lysine residues were identified to be within the coiled-coil domain (lys100, lys392) and three positions (lys10, lys471, lys482) occur outwith the coiled-coil domains. Panel-B shows the immunoblotting for immunoprecipitation of fraction 4 of cytoskeletal isolation from three cell lines i.e., HCT-116, Caco2, HT-29 [either untreated (C), or treated with butyrate (B) 5mM for 17 hours]. After separation of proteins gels were immunoprobed for K8 and three antibodies against lysine residues at potions 10, 471, and 482. To identify which fraction contains maximum K8 and acetylated lysines HCT-116 cells were treated with 5mM for 17 hours and then cytoskeletal isolation was performed for both treated and untreated cells. All four fractions were immunoprobed with K8 and three antiacetyle antibodies (panel C).

2.1 Cell Culture

Cells (HCT-116, HT-29, and Caco2) were cultured in DMEM (Dulbecco/Vogt-modified Minimal Essential Medium) containing 1 g/L D-glutamine, 4 mM L-glutamine, 110mg/L sodium pyruvate and 25 mM HEPES (all supplied by Gibco). Added to the DMEM was penicillin/streptomycin (Gibco) and 10% FCS (Biosera, Sussex, UK). Cells were incubated at 37 ^{0}C, 5% CO2 in humidified air**.**

2.2 *Cytoskeletal isolation*

Protocol for intermediate filament cytoskeletal isolation was as described by Herman *et al* (27). For cytoskeletal isolation from cell lines, either treated with butyrate (5 mM, 17 hours) or otherwise, adherent cells were washed in warm PBS (Oxoid, UK) containing 2 mM $MgCl_2$ and 50mM pefabloc (Roche, Welwyn garden City, UK). Low Detergent Buffer (0.5X PBS; 50 mM MOPS pH 7; 10 μM $MgCl_2$; 2μM

pefabloc; 1 mM EGTA; 0.15% v/v Triton x-100) was then added. After 90 seconds buffer was removed and cells were centrifuged and supernatant was removed which is membrane bound/soluble protein fraction (Fraction 1). Cold extraction was performed with High detergent Buffer (0.5X PBS; 50 mM MOPS pH 7; 10 μm $MgCl_2$, 2 μM Pefabloc; 1% v/v Triton X-100, 1 m NaCl) which yielded the cytoskeleton (Fraction 2). Remaining sample was centrifuged to yield the high salt soluble fraction (Fraction 3) as supernatant and the remaining pellet was the cytoskeletal proper (Fraction 4). Samples were analysed by immunoblotting. Primary antibodies used were anti K8 (mouse monoclonal Abcam, 9023) and in-house antibodies raised against acetylated lysine residues of K8: lys10 (rabbit), lys482 (rabbit), and lys471 (chicken) (for validations of antibodies see supporting information in Drake *et al* (23)). After SDS-Page and Western transfer [standard method as described previously (28)] the cross-reaction was visualized using HRP-conjugated secondary antibodies (Dako rabbit for lys10 & lys482, Dako chicken for lys471, and Dako mouse for K8) and Western Lightening Chemiluminescence reagent plus (PerkinElmer, Boston, USA). Imaging was undertaken with Chemigenius Bio-Imaging system (Syngene).

2.3 *Immunocytochemistry*

HCT-116 cells were seeded in 96-well plate at $3x10^3$ per well and then grown for 48 hours. Cells were then treated with 0-20 mM of SCFAs (butyric acid, propionic acid, valeric acid, or valproic acid; Sigma). Internal triplicates were used in each experimental pass. After 24 hours of treatment cells were fixed at -20 ^{0}C with 100% methanol for 5 minutes before immunocytochemical (ICC) staining. 100 μl of PBS was left in each well at the end of ICC staining. Plates were then sealed and sent for HCA.

During ICC primary antibodies used were same as in Western Blot (see section 2.2). Cells were dual immunostained for K8 and one of the three antibodies to normalize the acetylation intensity against the total K8 for each field while quantifying specific acetylation. All antibodies were diluted in digitonin according to Cellomics' HCA protocol. Secondary antibodies used were anti-mouse Alexa fluor 555-red for K8, anti-rabbit Alexa fluor 444-green for lys10 and lys482. Nuclei were stained with Hoechst 33342.

During HCA analysis, images were captured on an Arrayscan II (Cellomics) and analysed using the proprietary Arrayscan compartmental analysis algorithm. Regions of interest (ROIs), which corresponded to cells, were obtained based on the Hoechst nuclear staining profile. A mask was then created so that only cytoplasmic staining was measured, thereby excluding any non-specific nuclear fluorescent signal (23). The analysis was corrected for background fluorescence by using the program's spot thresholding algorithm. The data generated measured the total staining intensity of the identified spots for K8, acetylated lys10, acetylated lys482, and acetylated lys471. A single value for each well was obtained by averaging the total staining intensities across the ROI population for the well.

In a different experiment, to compare acetylation in dividing and non-dividing cells, Caco2 cells were grown for 48 hours and then treated with various butyrate concentrations for 24 hours. Same primary and secondary antibodies were used as described above and same ICC protocol was followed. All control experiments were analyzed for increase in acetylated lysines in mitotic cells by using HCA.

2.4 *Statistical analysis*

GraphPad Prism5 was used to calculate p values by using paired T test. Graphs were generated through GraphPadPrism5**.**

3. Results

3.1 *Keratin 8 is identified to have two isoforms which are differentially acetylated*

We have previously identified the five lysine residues which are acetylated in K8 (10) and further acetylation sites have been identified in a subsequent high-throughput study (24). Figure 1A shows the positions of these lysine residues relative to coiled-coil domains of K8. Three acetylation sites (lys10, lys471 & lys482) were found to be outside the coiled-coil domain at the N- and C-termini. Polyclonal antibodies have previously been described to these modified sites (23).

We first sought to establish whether K8 acetylation occurred in multiple colon cell lines. HCT-116, Caco2, and HT -29 were treated with butyrate (5 mM for 17 hours) and then insoluble fractions of treated and untreated cells were immunoprobed with antibodies against K8 and acetylated lysines (lys10, lys482, lys471) (Fig 1B). The panel indicates that lys10 and lys482 showed reactivity in a band corresponding to that of K8. Also, it is evident that K8 acetylation at lys482 was more marked in the higher molecular weight K8 band and lys10 acetylation was present in both forms of K8.

In order to determine in which fraction keratin 8 was most highly acetylated, fractions of HCT-116 cells were immunoprobed with K8, lys10, lys471, and lys482 (Fig 1C). K8 is most abundant in the insoluble fraction (fraction 4). The lower panels further demonstrate that most reactivity with anti-acetyl antibodies is seen in fraction 4 which demonstrates that K8 is acetylated when in the insoluble intermediate filament. Another interesting observation was that K8 appeared to exist as a doublet.

3.2 *SCFAs increase K8 acetylation in vitro*

Our previous work (10) suggested that butyrate can increase acetylation of K8. To demonstrate the effect of butyric acid and other SCFAs on K8 acetylation, HCT-116 colon cancer cells were treated with different SCFAs at five different concentrations and then the changes in K8 acetylation

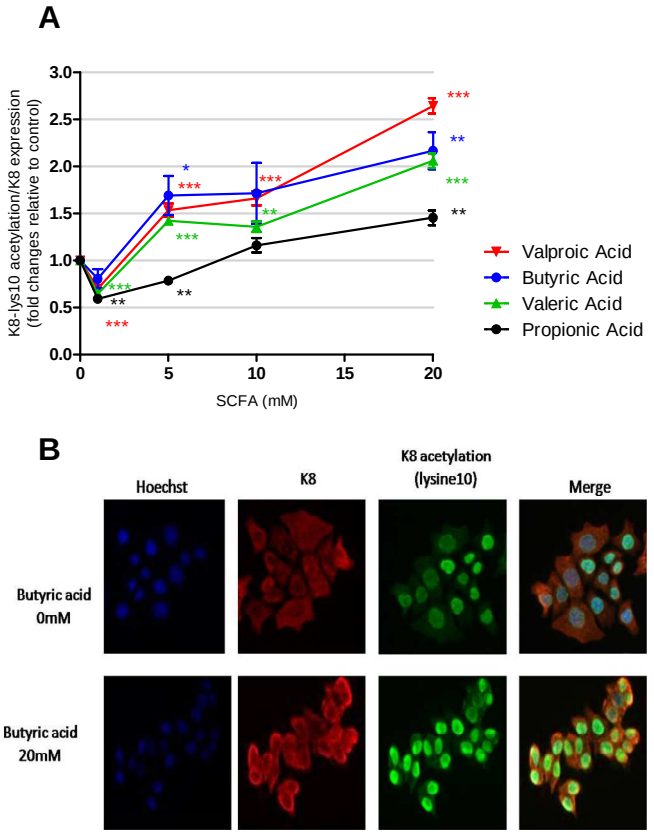

Figure 2. *Changes in K8-lys10 acetylation in response to SCFAs.* HCT-116 cells were grown for 48 hours and then treated for 24 hours with increasing doses of four different SCFAs (butyric acid, propionic acid, valeric acid, valproic acid). After 24 hours cells were fixed with cold methanol. For immunochemistry cells were treated with antibody against K8 (Abcam 9023) and antiacetyle antibody for acetylated lysine residue at position 10 (lys10). Binding of K8 antibody was detected with a secondary antibody (Alexa flour 555-red) and binding of lys10 was detected with another secondary antibody (Alexa Flour 444-green). Cell nuclei were stained with Hoechst. High Content Analysis (HCA) was performed on a Cellomics Arrayscan. Nine fields were scored for each concentration of individual SCFA treatment. Total K8 was quantified for each field along with the intensity of Alexa-444 staining. Lys10 acetylation was normalized against total K8 expression for each filed and then data was normalized to the control (no treatment). Figure2-A shows the fold changes in lys10 acetylation for each dose of SCFA compared to control. Data represents the mean ± SEM for three experiments with three internal repeats in each experiment. P values were calculated for lys10 acetylation at each SCFA concentration. *p<0.05, **p<0.01, ***p<0.001 relative to control (Paired t-test with GraphPad Prism5). Figure 2B shows the cells with or without treatment of butyric acid. Nuclei were localized with Hoechst (blue). Cytoplasmic staining of K8 (red) was colocalize with the anti-acetyl antibody (green) as shown by the yellowish merge in the last picture on the right.

were analysed by HCA. HCA was used to analyze the results which has already been proved to be an effective and quick way of quantify total K8 and K8 acetylation (23).

HCT-116 cells treatment with 1 mM of all SCFAs showed a significant decrease (p<0.01 for propionic acid and p<0.001 for valeric and valproic acid) in acetylation at lys10, except for butyric acid (Figure 2A). Exposure of HCT-116 cells to 5 mM of each SCFA caused a significant increase (p<0.05) in lys10 acetylation with all SCFAs. There was not a marked difference of response between SCFAs treatment at 5 mM. At 10 mM of each SCFA a plateau in the concentration response was reached. Maximum response was observed at 20 mM treatment for all SCFAs and valproic acid caused the largest increase (p<0.001) in acetylation at 20 mM among all SCFAs (Figure 2A).

The response in lys482 acetylation with SCFAs was not as profound as for lys10 acetylation (Figure 3A). At 1 mM concentration all SCFAs caused a significant decrease (p<0.05) in K482 acetylation except propionic acid. Valproic acid and valeric acid did not increase acetylation at any concentration when compared with control (0 mM). Propionic acid and butyric acid treated cells at 10 mM showed a significant increase in lys482 acetylation (p <0.01 for propionic acid and <0.001 for butyric acid). At 20 mM exposure, cells treated with propionic acid showed a significant increase (p<0.05) in lys482 acetylation when compared to the control but response with butyric acid was not significant at 20 mM.

Similar to response with lys10 & 482, lys471 also showed a significant decrease in acetylation with valproic acid

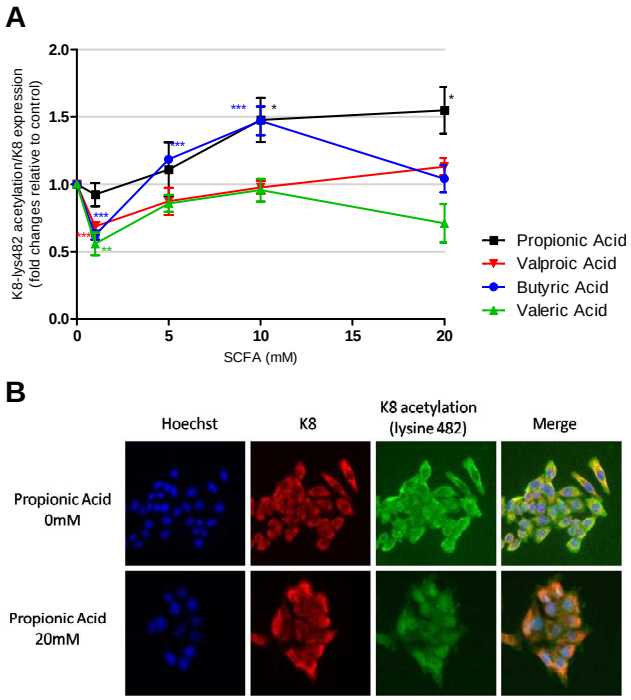

Figure 3. *Changes in K8-lys482 acetylation in response to SCFAs.* Figure3-A shows the fold changes in lys482 acetylation for each dose of SCFA compared to control. Data represents the mean ± SEM for three experiments with three internal repeats in each experiment. P values were calculated for lys10 acetylation at each SCFA concentration. *p<0.05, **p<o.01, ***p<0.001 relative to control (Paired t-test with GraphPad Prism5). Figure 3B shows the cells with or without treatment of propionic acid. Nuclei were localized with Hoechst (blue). Cytoplasmic staining of K8 (red) was colocalize with the anti-acetyl antibody (green) as shown by the yellowish merge in the last picture on the right.

($p<0.05$) and propionic acid ($p<0.01$) at 1 mM treatment but butyric acid and valeric acid increased lys471 acetylation at 1 mM (not statistically significant) (Figure 4). Propionic acid and valproic acid caused a decrease in lys471 acetylation at all concentrations except at 20 mM where valproic acid increased acetylation compared to control, although this was not statistically significant. Butyric acid caused a significant increase ($p<0.01$) in lys471 acetylation at 20 mM treatment.

Figure 5 shows changes in acetylation at lys10, lys482, and lys470 in response to each SCFA. It can be observed that following SCFA treatment. Lys10 acetylation consistently preceded the lys482 acetylation in butyric-, valeric- and valproic-acid treated cells while lys482 acetylation only preceded the lys10 acetylation in propionic acid-treated cells. This may suggests that K8 acetylation is generally hierarchical at the three but is not inconsistent with different HDACs (with differing inhibition profiles by the SCFA) are involved in governing different acetylation sites.

3.3 *Keratin 8 lys10 cell acetylation increases during mitosis*

Mitosis is an important step during cell division and also various factors could affect this process. We investigated whether there is a differential change in K8 acetylation during mitosis and compared the acetylation in both dividing and non-dividing cells. Interestingly, microscopy revealed that there was an increase in K8 acetylation in mitotic cells (Fig 6A). To study this further K8 acetylation was analysed by HCA, by gating cells with the greatest intensity of nuclear staining (Fig 6B). Mitotic cells were found to have a fivefold increase in lys10 acetylation by comparison with non-mitotic cells, whereas there was a more modest increase at lys482 and lys471 (Fig 6C).

4. Discussion

K8 has been shown to have many PTMs which could influence its structure and function. We previously showed that butyrate can increase K8 acetylation. In the present study we demonstrate the different sites of acetylation of K8 are modified differentially in response to treatment with different SCFAs, and to mitosis. We demonstrated that HCB is a useful tool to study K8 acetylation.

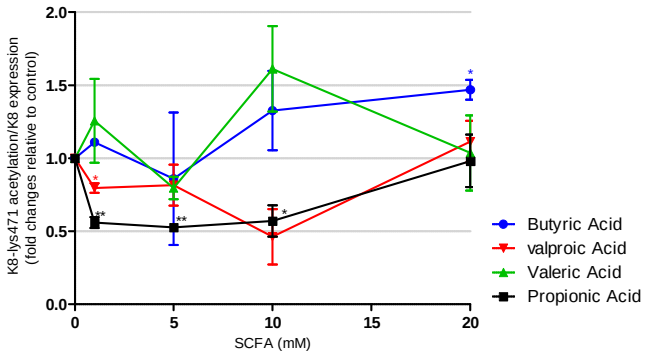

Figure 4. *Changes in K8-lys471 acetylation in response to SCFAs.* The fold changes in lys471 acetylation for each dose of SCFA compared to control. Data represents the mean ± SEM for one experiment with three internal repeats. P values were calculated for lys471 acetylation at each SCFA concentration. *p<0.05, **p<o.01, ***p<0.001 relative to control (Paired t-test with GraphPad Prism5).

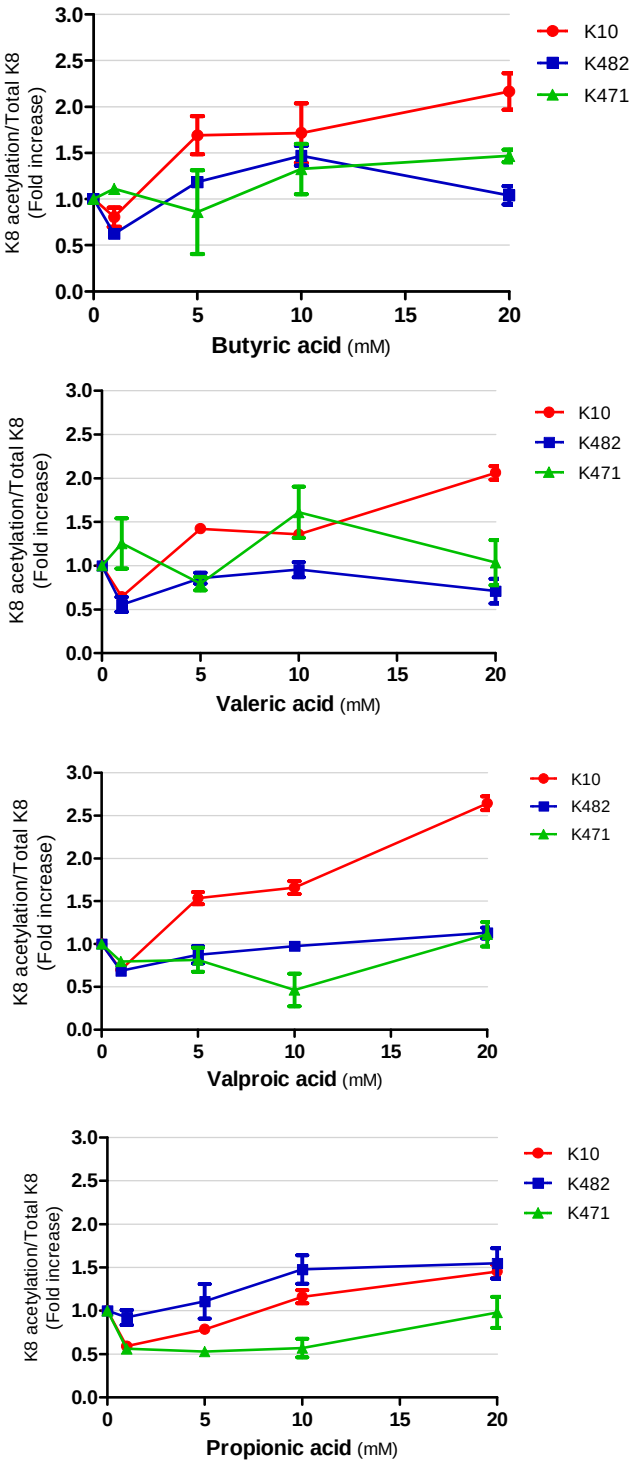

Figure 5. *Differential response of K8 acetylation to short chain fatty acids.*

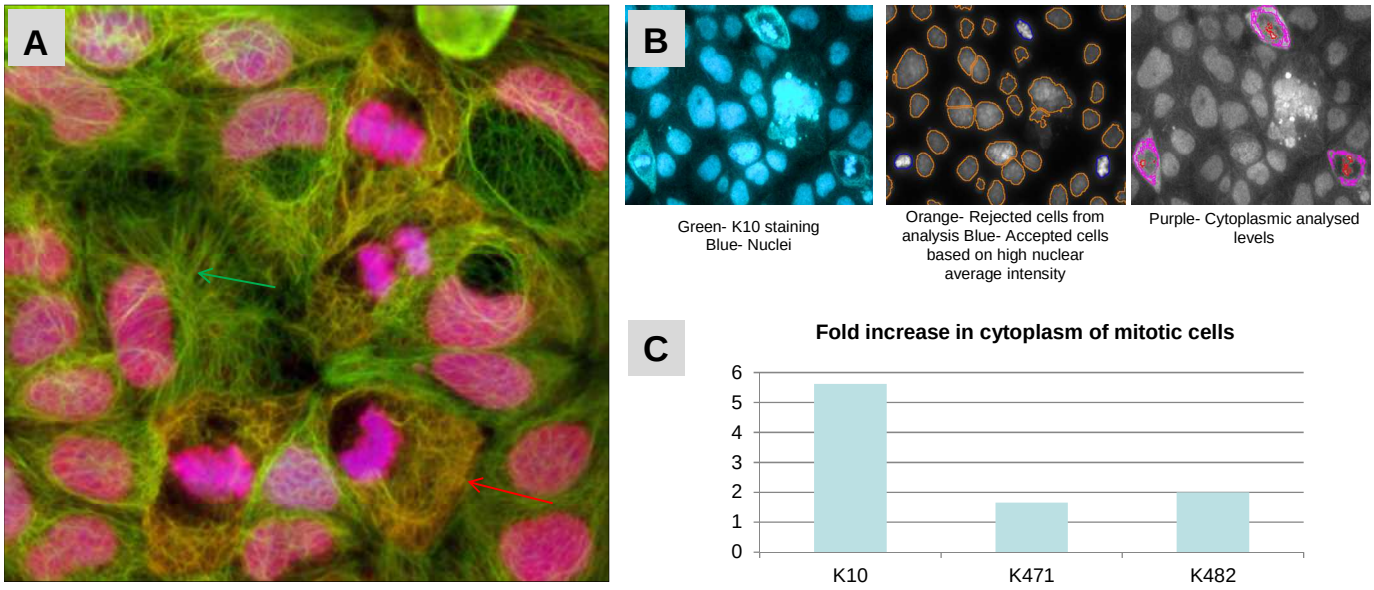

Figure 6.*Keratin 8 is more acetylated in mitotic cells.* Caco2 cells were grown for 48 hours and then treated with butyrate for 24 hours before fixing in ice cold methanol. Immunocytochemical staining for K8 and anti lysine antibodies was undertaken. Top picture (Fig 6A) shows that the filaments (K8) are greenish in non dividing cells (as shown with a green arrow) but the cell with a dividing nucleus (mitotic cell) shows the more yellowish (as indicated by a red arrow) fibrous network (K8) which indicated more acetylated K8 in mitotic cells. Figure 6B shows the scheme how acetylation was measured only in mitotic cells by rejecting the non-dividing cells. All control experiments were analysed for increase in acetylated lysines in mitotic cells (Figure 6C).

When the insoluble fractions of various colon cancer cell lines were immunoblotted we identified two isoforms of K8 which were differentially acetylated. This suggests that various isoforms are differentially modified. As lys10 acetylation occurs on the shorter form, and lys482 appears strongly acetylated in the short form and weakly acetylated in the longer form there is no reason to invoke loss of either end of the protein in production of these isoforms, which may potentially arise as a consequence of alterations in mass of the protein due to different burdens of post-translational modification.

Among various HDAC inhibitors, valproic acid has some attractive characteristics which make it suitable for a potential candidate to treat CRC. It has already been used for long time as an anticonvulsant and therefore its pharmacokinetic properties have been tested. It is available orally, well tolerated and has a longer *in vivo* half life compared with other HDAC inhibitors (29, 30). We showed, in this experiment, that all four studied SCFAs at 20 mM treatment (butyric acid, propionic acid, valeric acid and valproic acid) increase K8 acetylation at lys10. These observations again reinforce the theory that butyrate and other SCFAs can change the cell homeostasis through increase in K8 acetylation. Although the consequences of K8 acetylation are beyond the scope of this particular study but as mentioned in our previous report this acetylation could ultimately affect the cell apoptosis or could affect the polymerisation of cytoskeleton.

In conclusion, the present work provides evidence that acetylation is another PTM for K8 and that there are, at least, two isoforms of K8 which are differentially acetylated. SCFAs increase K8 acetylation and maximum lys10 acetylation is induced with valproic acid and for lys482 propionic acid produced maximal acetylation.

The results were achieved using a high content biology approach. Through application of this methodology, it was possible to measure the total intensity of K8 expression and also changes in K8 acetylation in order to allow us to find the relative increase in K8 acetylation. The technique was unique in allowing us to investigate both quantitatively and qualitatively the spatial alterations in keratin acetylation, for example in mitotic cells, representing a significant advantage over either microscopy or flow cytometry. This technique can successfully be applied to study acetylation response with various HDAC inhibitors. Future studies should address the relationship between K8 acetylation and structure and function.

Acknowledgements

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