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Differentiation of Human Colon Adenocarcinoma Cells Alters the Expression and Intracellular Localization of Annexins A1, A2, and A5

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Abstract Butyrate induces differentiation and alters cell proliferation in intestinal–epithelial cells by modulation of the expression of several genes. Annexins are a superfamily of ubiquitous proteins characterized by their calcium-dependent ability to bind to biological membranes; their involvement in several physiological processes, such as membrane trafficking, calcium signaling, cell motility, proliferation, and differentiation has been proposed. Thus, we have analyzed changes in annexin A1 (AnxA1), annexin A2 (AnxA2), and annexin A5 (AnxA5) levels and localization in human colon adenocarcinoma cells differentiated by butyrate treatment or by culture in glucose-free inosine-containing medium. The acquired differentiated phenotype increased dipeptidyl peptidase-IV (DPP-IV) expression and alkaline phosphatase (ALP) activity, two well known brush border markers. Butyrate induces cell differentiation and growth arrest in BCS-TC2, BCS-TC2.2, HT-29, and Caco-2 cells, increasing the levels of AnxA1 and AnxA5, whereas AnxA2 decreases except in Caco-2 cells. Inosine-differentiated cells present increased amounts of the three studied annexins, as occurs in spontaneously differentiated Caco-2 cells. AnxA2 down-regulation is not due to proteasome activation and seems to be related to the butyrate-induced cell proliferation arrest; AnxA1 and AnxA5 expression is growth-state independent. AnxA1 and AnxA5 are mainly found in the cytoplasm while AnxA2 is localized underneath the plasma membrane in cell-to-cell contacts. Butyrate induces changes in subcellular localization towards a vesicle-associated pattern. Human colon adenocarcinoma cell differentiation is associated with an up-regulation of AnxA1, AnxA2, and AnxA5 and with a subcellular relocation of these proteins. No correlation between annexin levels and tumorigenicity was found. Up-regulation of AnxA1 could contribute to the reported anti-inflammatory effects of butyrate in colon inflammatory diseases. J. Cell. Biochem. 94: 178–193, 2005.

Key words: annexin; butyrate differentiation; inosine; proteasome

Luminal nutrition is important for the maintenance of gastrointestinal mucosa function and structure, contributing to the control of intestinal crypt architecture, cell proliferation, rates of cell migration, and apoptosis. Short-chain fatty acids, which are produced in the intraluminal compartment through colonic bacterial fermentation of dietary fiber and resistant starches [Topping and Clifton, 2001], regulate intestinal–epithelial cell growth and differentiation, playing a role in some gastrointestinal diseases [Andoh et al., 2003]. Among them, butyrate not only is the preferred energy source for colonocytes but it is also required for the maintenance of normal colonic epithelial cell development, and is supposed to control colonocyte functions. In fact, butyrate has been described to be one of the most potent regulators of cell growth and differentiation and could account for part of the protective effect of dietary fiber in colon carcinogenesis [Augenlicht et al., 2002]. It has also been implicated in

Abbreviations used: ALP, alkaline phosphatase; AnxA1, annexin A1; AnxA2, annexin A2; AnxA5, annexin A5; BSA, bovine serum albumin; DAPI, 4′-6-diamidino-2-phenylindole; DPP-IV, dipeptidyl peptidase-IV; FCS, fetal calf serum.

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protection against inflammatory bowel disease, ulcerative colitis, and Crohn's disease [Andoh et al., 2003]. For all these reasons, butyrate has been considered for therapeutic purposes: to suppress colon inflammation and for the treatment of colorectal cancer.

The molecular basis of butyrate therapeutic activities seems to be related to its ability to modulate gene expression, signal transduction, and protein degradation pathways [Yin et al., 2001; Augenlicht et al., 2002; Andoh et al., 2003]. Some of the butyrate-regulated genes are involved in the modulation of cell-cycle progression, apoptosis, and differentiation [Coradini et al., 2000; Tabuchi et al., 2002]. It has been described that nucleotides may also affect proliferation and maturation of intestinal epithelium. Some reports show the differentiation of colon adenocarcinoma cells towards an enterocytic phenotype by culture in the absence of glucose and using inosine as carbon source [Wice and Gordon, 1995; Ancillotti et al., 2003]. Cell growth and viability are not impaired under these conditions. Besides, the endogenous purine inosine has been shown to exert anti-inflammatory effects in several human cell types [Marton et al., 2001]. Additionally, spontaneous differentiation may be achieved in specific colon adenocarcinoma cell lines as Caco-2 by long-term culture in post-confluent conditions [Hara et al., 1993; Engle et al., 1998].

Annexins constitute a superfamily of proteins characterized by their ability to bind acidic phospholipid-rich biological membranes in the presence of calcium [Gerke and Moss, 2002]. Up to date, 12 annexin genes have been reported in humans [Fernandez and Morgan, 2003]. All these proteins present a homologous core structure and a highly variable N-terminus that is probably responsible for the regulation of the specific functions of each annexin [Turnay et al., 2003]. Their physiological roles are not clear but they seem to be involved in several processes as inflammation, membrane trafficking and calcium signaling, among others [Gerke and Moss, 2002; Turnay et al., 2003]. On this idea, annexin A1 (AnxA1) has been described as a potent endogenous anti-inflammatory protein, and antibodies against this protein have been found in patients with ulcerative colitis and Crohn's disease [Perretti and Gavins, 2003]. Additionally, AnxA1 and annexin A2 (AnxA2) are the main targets of some protein tyrosine kinases and protein kinase C [Gerke and Moss, 2002]. Moreover, mitogens that activate protein kinase C, as phorbol esters, not only induce phosphorylation of AnxA1 but also increase its expression and promote its nuclear translocation [Kim et al., 2003]. Several members of this family of proteins, including AnxA1, AnxA2, and annexin A5 (AnxA5), are differentially expressed during development, cell proliferation, and differentiation processes [Della Gaspera et al., 2001; Turnay et al., 2002], or are even related to tumor progression and malignancy [Gerke and Moss, 2002; Xin et al., 2003].

In this study, we have analyzed AnxA1, AnxA2, and AnxA5 protein levels in human colon adenocarcinoma cell lines and the modifications associated with butyrate- and inosine-induced differentiation. We have used several established cell lines with quite different behavior in culture mainly regarding tumorigenicity and ability to differentiate. Thus, they constitute a good model for studying annexin expression during cell differentiation.

MATERIALS AND METHODS

Cell Lines and Cell Culture

BCS-TC2 cells were obtained in our laboratory from a poorly differentiated human colon adenocarcinoma; they present a low differentiation degree and show almost null tumorigenicity in vivo [Turnay et al., 1990]. Co-injection of these cells with matrigel and in vivo passage in athymic mice allowed the establishment of the BCS-TC2.2 cell subline. These cells present intrinsic tumorigenic potential and a lower differentiation degree than the parental ones [López-Conejo et al., 1996]. The HT-29 cell line (ATCC HTB38), under standard culture conditions, is mainly composed of poorly differentiated cells and a small percentage of either columnar absorptive or mucus-secreting differentiated cells [Blottiere et al., 1993]. On the other hand, Caco-2 cells (ATCC HTB37) present features of differentiated cells, including the ability to form domes in post-confluent culture and even to undergo further spontaneous differentiation with time in culture [Hara et al., 1993; Engle et al., 1998].

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (10% for Caco-2 cells) heat-inactivated fetal calf serum (FCS), penicillin (50 IU/ml), streptomycin (50 μg/ml), and glutamine (300 μg/ml). Cell subculture was performed by trypsiniza-

tion; 1.5×10^4 cell/cm^2 were usually plated onto tissue culture dishes with medium changes every second day. Butyrate (Sigma, Alcobendas, Spain) treatment was performed on exponentially growing cells, which were maintained in the presence of this agent for 4 days unless otherwise stated.

Cell proliferation was assessed by determination of the cell number after detachment by trypsinization. For this purpose, 3.4×10^4 cells were seeded into 9.5 cm^2 cell culture plates and maintained in standard medium for 2 days. Culture media was then replaced by fresh one containing 2, 5, 10% FCS or 5% FCS plus 4 mM butyrate.

Cell differentiation with inosine (Sigma) was induced by continuous culture of the cells (at least 2 months) in glucose-free DMEM (Biochrom AG, Berlin, Germany) supplemented with 2.5 mM inosine and containing 10% dialyzed-serum. Cells were passaged every week and used between passages 8 and 10. The differentiated phenotype is maintained as long as the specific culture conditions are kept. Spontaneous differentiation of Caco-2 cells was achieved maintaining post-confluent cultures for at least 1 week under standard culture conditions without trypsinization.

In order to achieve proteasome inhibition, semiconfluent cell cultures were preincubated for 30 min in the presence of 5 μM MG101 (*N*-acetyl-Leu-Leu-NorLeu-AL) or MG132 (*N*-CBZ-Leu-Leu-Leu-AL), both from Sigma. Cells were further incubated with the corresponding proteasome inhibitor for 4 days in the absence or in the presence of 4 mM butyrate.

Conditioned media were obtained from near confluent cultures after washing twice with PBS and incubation for 2 days in supplemented DMEM without serum, in the absence or presence of butyrate. After centrifugation, media were 300-fold concentrated before Western blot analysis.

ALP Activity

Activity of the colonic-epithelium differentiation marker ALP was measured in cell extracts obtained as previously described [Navarro et al., 1997] using a commercially available Sigma diagnostics kit (procedure 245). One unit of enzyme activity is defined as 1 μmol of substrate hydrolyzed per minute at 37°C. Protein content was determined using the D_C Protein Assay (Bio-Rad, Madrid, Spain).

Western Blot Analysis

Cell homogenates were analyzed by PAGE–SDS after heat denaturing in the presence of 5% β-mercaptoethanol. Proteins were transferred to nitrocellulose membranes and analyzed by Western blot as described elsewhere [Turnay et al., 2002]. Development was performed using the Enhanced Chemiluminiscence system from Amersham-Pharmacia-Biotech (Buckinghamshire, UK). Films were scanned and a densitometric analysis was performed obtaining volumograms on a photodocumentation system from UVItec (Cambridge, UK) and using the UVIBand V.97 software [Turnay et al., 2002].

The monoclonal anti-human AnxA1 antibody was from the DSHB (University of Iowa, Iowa City, IA). Rabbit anti-human AnxA5 polyclonal antibody was obtained in our laboratory. Monoclonal antibodies against human AnxA2, HSP70i, and p53 were from Transduction Laboratories (BD Biosciences, San Diego, CA), Stress Gen (Victoria, BC, Canada) and from Sigma, respectively. Peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad and peroxidase-conjugated goat anti-mouse IgG from Pierce (Bonn, Germany). The absence of cross-reactivity among the different anti-annexin antibodies was verified in our laboratory by Western blot.

Immunofluorescence Microscopy

Immunofluorescent staining was performed on cells growing on culture plastic in the absence or in the presence of butyrate for 4 days. Cells were fixed in cold methanol and immunostained as previously described [Gutiérrez-López et al., 2003]. The specific anti-human AnxA1 monoclonal antibody [Pepinsky et al., 1990] was kindly provided by Dr. B.C. Pepinsky (Biogen, Inc., Cambridge, MA); antibodies against AnxA2 and AnxA5 were the same as described above for Western blot. Fluorescein-conjugated secondary anti-rabbit or anti-mouse IgG were purchased from Chemicon (Temecula, CA). Counterstaining was performed with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma) at 1 μg/ml. Samples were mounted with Fluoroguard (Bio-Rad) and viewed under an Axioplan 2 photomicroscope (Carl Zeiss, Oberkochen, Germany); images were captured by a SPOT slider 2 digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Each channel was recorded independently, and pseudocolor images were generated and superimposed. Digital

images were processed with the MetaMorph 5.0 software (UIC, Downingtown, PA).

Single-stained preparations were analyzed by confocal microscopy using a Bio-Rad Laboratories MRC-1024 Laser Scanning microscope equipped with an Eclipse TE300 inverted microscope (Nikon, Düsseldorf, Germany).

FACS Analysis

Cells were detached by trypsinization and allowed to recover for 30 min in complete culture medium. After washing twice with PBS, 5×10^5 cells were resuspended in 500 μl PBS containing 0.1% bovine serum albumin (BSA). After addition of 10 μl of the appropriate dilution of R-phycoerythrin-conjugated mouse anti-human dipeptidyl peptidase-IV (DPP-IV or CD-26) monoclonal antibody (PharMingen/BD Biosciences), the cell suspension was incubated for 1 h at 37°C in the dark. Cells were again washed twice with PBS containing 0.1% BSA and finally resuspended in 500 μl and analyzed in a FACScan (Becton-Dickinson, San José, CA), as described [López-Conejo et al., 1996].

Statistical Analysis

The differences between the mean values were analyzed with SigmaPlot v8.02 (SPSS, Chicago, IL) and using Student's *t*-test; statistical significance was considered to be achieved at the $P<0.05$ level.

RESULTS

AnxA1, AnxA2, and AnxA5 Protein Expression

Annexin protein levels were analyzed in the different human colon adenocarcinoma cells 24 h after seeding. Figure 1 shows the relative basal annexin levels in the four cell lines. BCS-TC2 cells and the tumorigenic subline BCS-TC2.2 present the highest AnxA1 content; a lower amount is detected in HT-29 cells and almost no expression appeared in Caco-2 cells, both of them tumorigenic. AnxA2 follows a similar pattern, with almost no expression in Caco-2 cells; however, the highest content is detected for HT-29 and BCS-TC2.2 cells, being lower for BCS-TC2 (around 50%). AnxA5 levels are quite similar among the cell lines except for Caco-2 cells, where AnxA5 levels are less than 50% of those corresponding to the other cell lines.

The localization of annexins in confluent cultures was analyzed by confocal laser microscopy. Figure 2 shows micrographs corresponding to BCS-TC2 cells. Staining of AnxA1 is mainly observed in the cytoplasm and at some areas of the plasma membrane in contact with

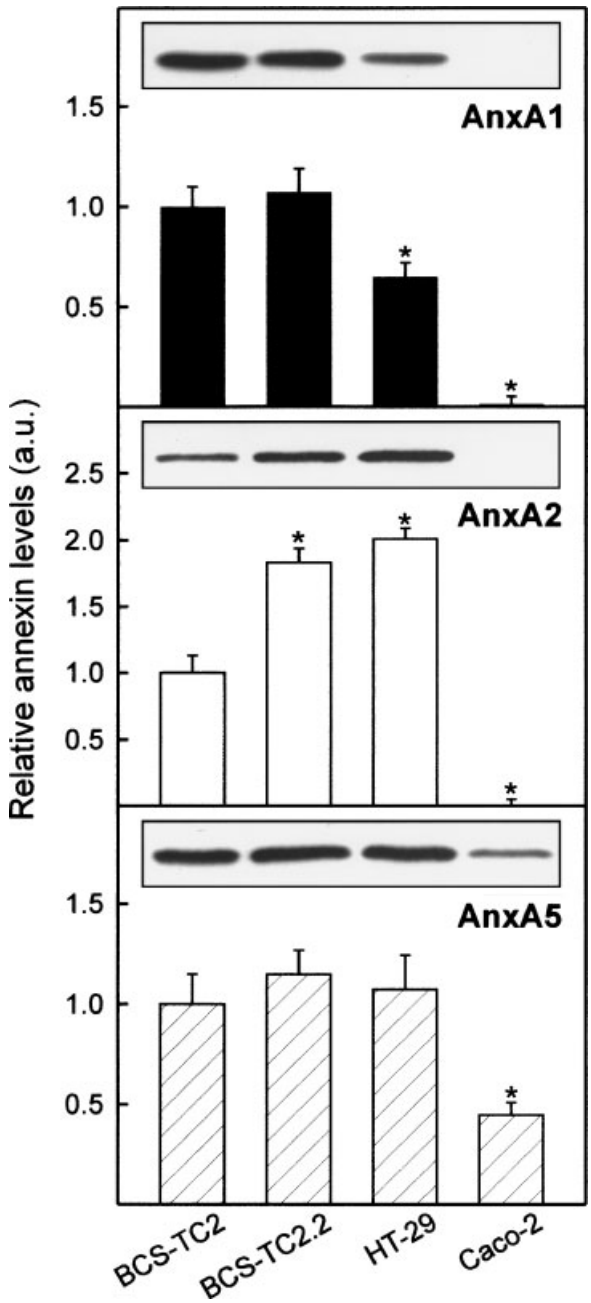

Fig. 1. Expression of annexin A1 (AnxA1), annexin A2 (AnxA2), and annexin A5 (AnxA5). Protein levels were analyzed 24 h after seeding the cells by Western blot of cell homogenates (15 μg protein). In each panel a representative blot is shown. At least five different independent samples were analyzed per cell line. Data (mean ± SD) are represented in arbitrary units (a.u.) and normalized to the intensity of the band corresponding to the amount of each annexin in BCS-TC2 cells. The statistical significance of the differences versus the levels in BCS-TC2 cells is indicated by an asterisk ($P < 0.01$).

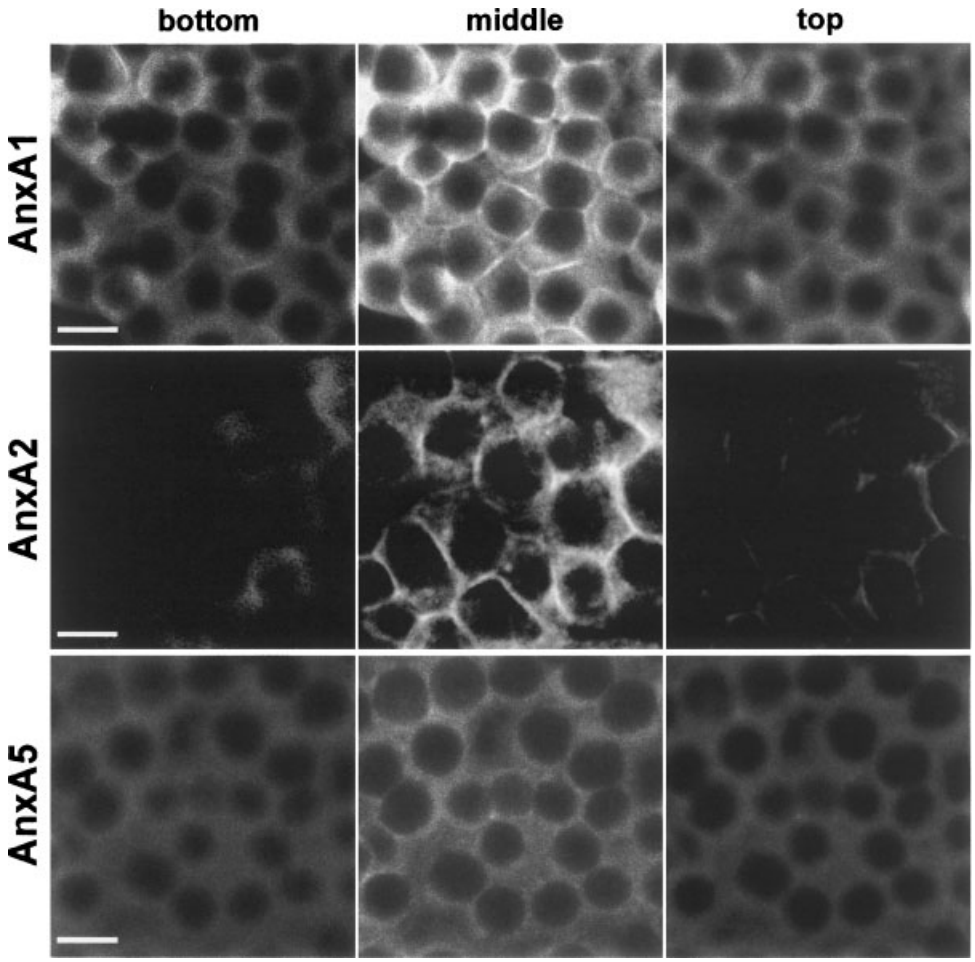

Fig. 2. Confocal laser microscopy analysis of the expression of AnxA1, AnxA2, and AnxA5. BCS-TC2 cells were grown for 4 days in standard medium, fixed, subjected to immunostaining, and analyzed by confocal microscopy. Images from the **bottom**, **middle**, and **top** planes are shown. Scale bar = 25 μm.

neighboring cells, apparently in regions of cell-to-cell interactions (Fig. 2, middle plane). AnxA2 is found predominantly at the plasma membrane, and a particular enrichment at the sites of cell-to-cell contacts is detected with only a faint cytoplasmic staining close to the plasma membrane in some cells; in contrast, AnxA5 is homogeneously distributed in the cytoplasm. Some cells present a perinuclear AnxA1 and AnxA5 staining. No significant nuclear localization was observed for the three studied annexins.

Effect of Butyrate Treatment on Cell Differentiation and Annexin Expression

Butyrate treatment induces a concentration-dependent increase in ALP activity in the studied cell lines (Fig. 3), but the extent of the increase significantly varies among them. The tumorigenic BCS-TC2.2 cells show the lowest effect, with only around 5-fold increase in ALP activity after 4 days exposure to 2 or 4 mM butyrate. The effect is higher in their non-tumorigenic parental cells (BCS-TC2) and in HT-29 cells (8.4- and 28-fold increase at 4 mM butyrate treatment, respectively). Caco-2 cells present not only the highest basal ALP activity levels (34.8 ± 2.3 mU/mg) but also show the highest increase with butyrate treatment: 38- and 136-fold increase after 4 days treatment with 2 and 4 mM butyrate, respectively.

Annexin expression was analyzed by Western blot and further densitometric analysis in exponentially growing cells after 4 days treatment with 2, 4, and 8 mM butyrate (Fig. 4). AnxA1 levels significantly rise in all cell lines but a different behavior is detected among them. Whereas a continuous increase in AnxA1 levels is detected in Caco-2 (24-fold) and BCS-TC2.2 cells (7.8-fold) with butyrate concentration up to

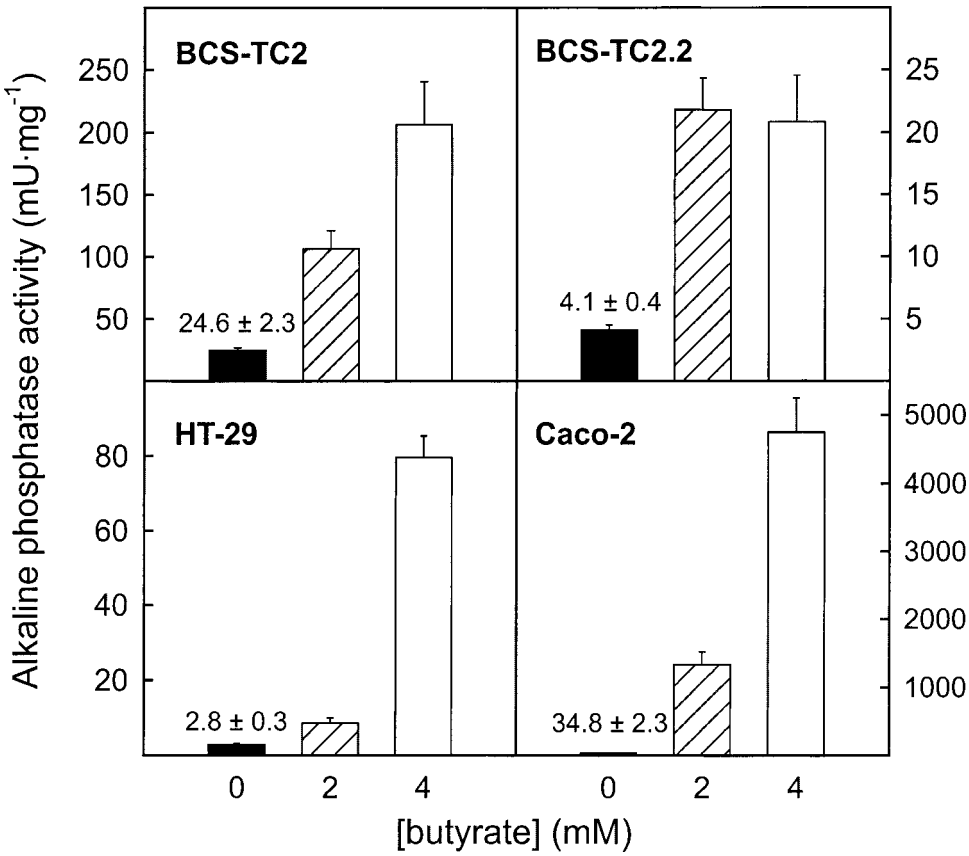

Fig. 3. Effect of butyrate treatment in alkaline phosphatase (ALP) activity. Cells were incubated for 4 days in standard culture media in the absence or presence of butyrate. Control activities in mU/mg are shown in each panel. At least four independent determinations were performed under each experimental condition; data represent mean values (±SD). In all experimental conditions, incubation with butyrate induced a statistically significant increase ($P < 0.01$) in comparison to the corresponding control cultures.

8 mM, BCS-TC2 and HT-29 cells show a maximum at 2 mM (3.7-fold) or 4 mM butyrate (4.8-fold), respectively, decreasing thereafter. AnxA5 expression is induced by butyrate to a lower extent than AnxA1; the possible cytotoxicity at high butyrate concentrations does not apparently affect AnxA5 protein levels as occurs with AnxA1, at least in BCS-TC2 and HT-29 cells. AnxA2 follows a completely different pattern; a significant concentration-dependent decrease in its levels is detected up to 8 mM butyrate in BCS-TC2 (24-fold), BCS-TC2.2 (5-fold), and HT-29 cells (67-fold), while a high increase is observed in Caco-2 cells (39-fold at 8 mM butyrate).

Effect of Proteasome Inhibition on AnxA2 Protein Levels in BCS-TC2 Cells

Proteasome activation could be responsible for the decrease in AnxA2 expression with butyrate treatment. To investigate this possibility, we have analyzed the effect of butyrate in AnxA2 levels in the absence or presence of the proteasome inhibitors MG132 and MG101. MG132 strongly affects cell viability after 4 days incubation even in the absence of butyrate, while this cytotoxic effect is almost negligible for MG101. Figure 5 shows the effect of butyrate in the levels of AnxA2, HSP70i, and p53 in BCS-TC2 cells in the absence or presence of MG101. Butyrate induces a decrease in the amounts of the three proteins in the absence of the proteasome inhibitor (2.6-, 2.0-, and 2.1-fold in AnxA2, HSP70i, and p53, respectively). When MG101 is present, butyrate does not induce any significant change in HSP70i or p53 levels, but the decrease in AnxA2 is still observed (2.5-fold).

Induction of Cell Differentiation by Alternative Procedures

In order to confirm the relationship between cell differentiation and annexin expression, we

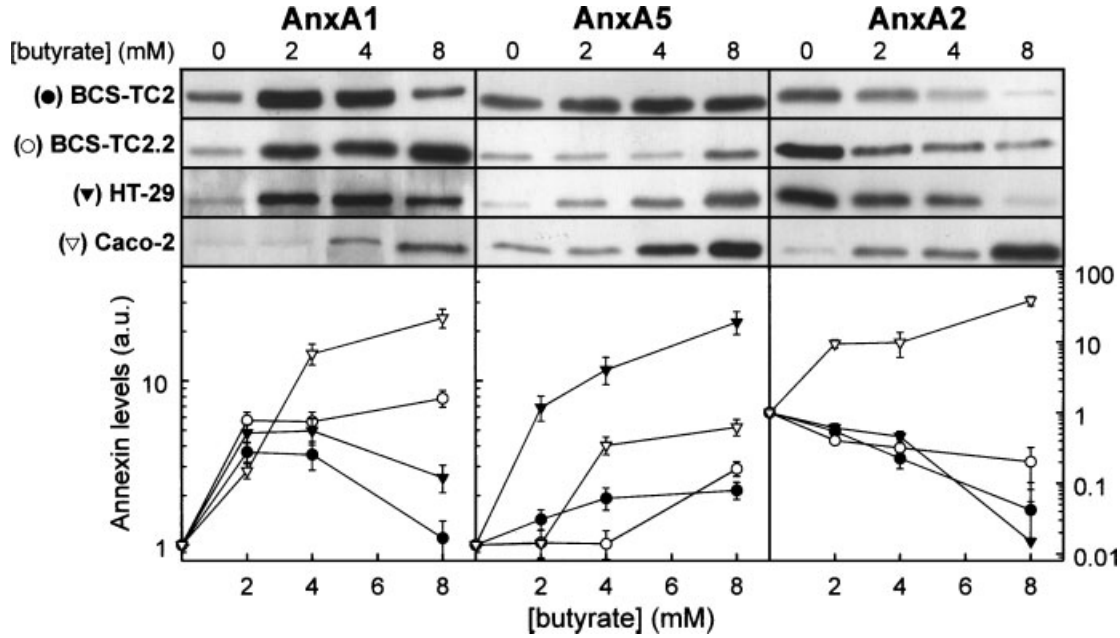

Fig. 4. Effect of butyrate treatment in annexin expression. Cells were incubated for 4 days in standard culture media in the absence or presence of butyrate; protein levels were analyzed by Western blot (15 μg protein). The **top** panels show representative blots for each annexin in the four cell lines. The **lower** panels show the densitometric analysis of samples from at least three independent experiments. Data are expressed in arbitrary units (a.u.; mean ± SD) after normalization versus the values obtained in the absence of butyrate for each annexin and cell line.

have induced this process in the human colon adenocarcinoma cells by alternative procedures. Cells were cultured under permissive differentiation conditions: inosine-supplemented glucose-free medium or, for Caco-2 cells, long-term culture in post-confluent state. Under these conditions, cells acquire a more differentiated phenotype compared to those growing in standard culture conditions. The brush-border-membrane hydrolase DPP-IV is one of the typical markers of differentiation [Yoshioka et al., 1991] that increases during inosine-induced differentiation. FACS analysis of cells differentiated under the above mentioned conditions clearly shows an increase in DPP-IV expression on the cell surface as observed from the peak shifts and the higher mean fluorescence intensity values (Fig. 6).

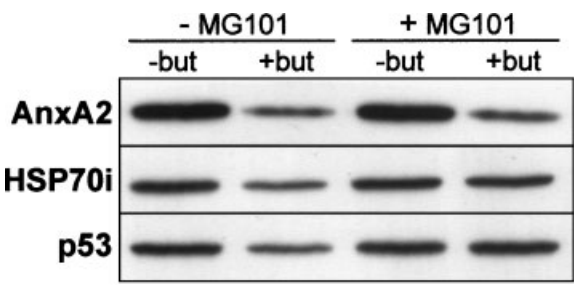

Fig. 5. Effect of proteasome inhibition in the modulation of AnxA2, HSP70i, and p53 expression by butyrate. BCS-TC2 cells were incubated for 4 days in standard culture medium without or with 4 mM butyrate and in the absence or presence of the proteasome inhibitor MG101. Cell lysates (15 μg protein) were analyzed by Western blot.

In order to analyze annexin levels, control and inosine-differentiated cells were simultaneously seeded into culture dishes, maintained in culture for 4 days, and cell homogenates were subjected to Western blot. Figure 7 shows that the differentiation of cells induced by glucose-deprivation in the presence of inosine also increases AnxA1 expression, as detected in butyrate-differentiated cells; protein levels increase 2.2-, 3.4-, and 3.1-fold in BCS-TC2, HT-29, and Caco-2 cells, respectively. AnxA5 levels also increase (2.0-fold in BCS-TC2 cells, 4.9-fold in HT-29 cells, and 1.8-fold in Caco-2 cells), showing a similar behavior to that obtained in butyrate-treated cells. Regarding AnxA2, Caco-2 cells present a 2.8-fold increase in its levels in the differentiated state. On the other hand, BCS-TC2 cells show almost no variation in the amount of this annexin, and only a slight increase is detected in HT-29 cells, in contrast with the significant decrease observed when these cells were differentiated via butyrate treatment (Fig. 4). The annexin expression pattern with inosine-differentiation in BCS-TC2.2

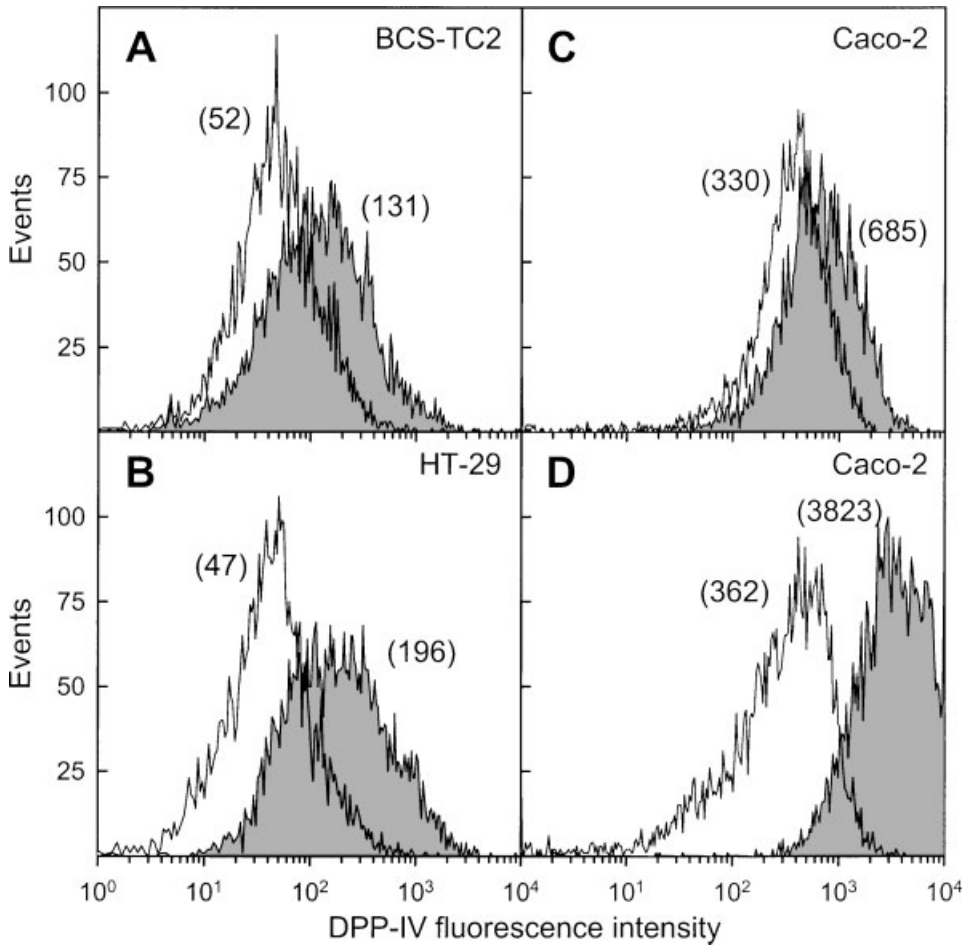

Fig. 6. Expression of dipeptidyl peptidase-IV (DPP-IV) in inosine-differentiated cells and spontaneous-differentiated Caco-2 cells. DPP-IV expression was analyzed in control (white) and inosine-differentiated (gray) cells (**A**–**C**) or spontaneous-differentiated (gray) Caco-2 cells (**D**) by flow cytometry using an R-phycoerythrin-conjugated specific antibody. Geometric mean fluorescence intensity values are indicated in brackets.

cells is almost identical to that of their parental cells.

Spontaneous differentiation of post-confluent Caco-2 cells was achieved according to morphological features (e.g., dome formation) and to a significant increase in DPP-IV expression (Fig. 6D) and ALP activity (from 38.8 ± 1.6 to 171.4 ± 4.5 mU/mg). Annexin expression was analyzed in these differentiated cells by Western blot (Fig. 8) and, again, a significant increase in AnxA1, AnxA2, and AnxA5 protein levels accompanies cell differentiation of Caco-2 cells.

Effect of Butyrate and Serum on Cell Growth and Annexin Expression

Taking into account that annexin expression may be dependent on the cell-growth state, the antiproliferative activity of butyrate may be responsible for the changes in the levels of these proteins. Thus, we have analyzed the influence of the cell growth rate (modifying serum concentration or adding 4 mM butyrate) in annexin expression.

Figure 9 shows the effect of 4 mM butyrate on BCS-TC2 and HT-29 cell growth patterns compared to control cultures, both in the presence of 5% serum. In both cell lines, butyrate induces a reduction in the cell growth rate with a decrease in cell number that can be observed after 4 days in culture in the presence of this agent. After 10 days in culture, the number of cells decreases 4.2- and 2.7-fold in BCS-TC2 cells and HT-29, respectively.

Additionally, we have analyzed the influence of serum concentration on BCS-TC2 cell growth

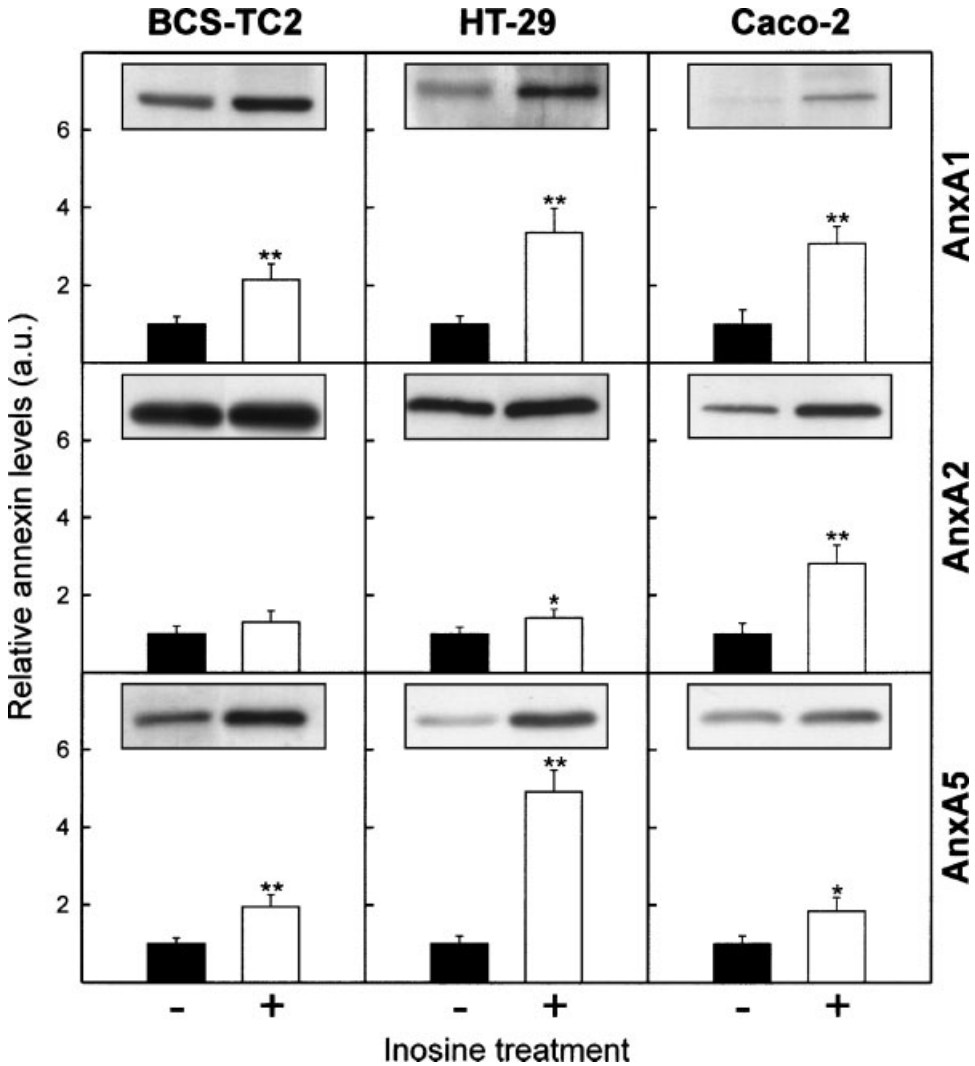

Fig. 7. Annexin expression in inosine-differentiated cells. Cell lysates (15 μg protein) of subconfluent cultures of control cells (−) and inosine-differentiated cells (+) were analyzed by Western blot. In each panel a representative blot is shown. At least three different culture plates were used per cell line and treatment, and were analyzed independently. Data (mean ± SD) are represented in a.u. and normalized to the intensity of the band of each annexin in the corresponding control cells. $^{*}P < 0.05$; $^{**}P < 0.01$.

(Fig. 9). These cells are able to proliferate even at low serum concentrations (2%), although the proliferation rate is clearly dependent on serum concentration at least up to 10%.

Figure 10 illustrates annexin protein levels along BCS-TC2 cell growth. We have examined annexin amounts at different incubation times in the presence of 2, 5, and 10% serum. No significant differences are found in the expression of AnxA1 and AnxA5 with serum concentration or with time in culture. In contrast, AnxA2 levels show a clear dependence with culture time even though there is no influence at each time point with serum concentration. Thus, AnxA2 levels increase 3.8- and 5.8-fold from day 6 to day 8 or 10, respectively.

Effect of Butyrate in Annexin Secretion

Annexin secretion and the influence of butyrate in this process have been analyzed as well. Conditioned media were obtained after growing cells for 2 days in culture medium without serum in the absence or in the presence of 4 mM butyrate. Under these experimental conditions, no apoptotic features (loss of membrane asymmetry or cell detachment) are observed. As shown in Figure 11, AnxA1, AnxA2, and AnxA5 are secreted by the four cell lines, although very

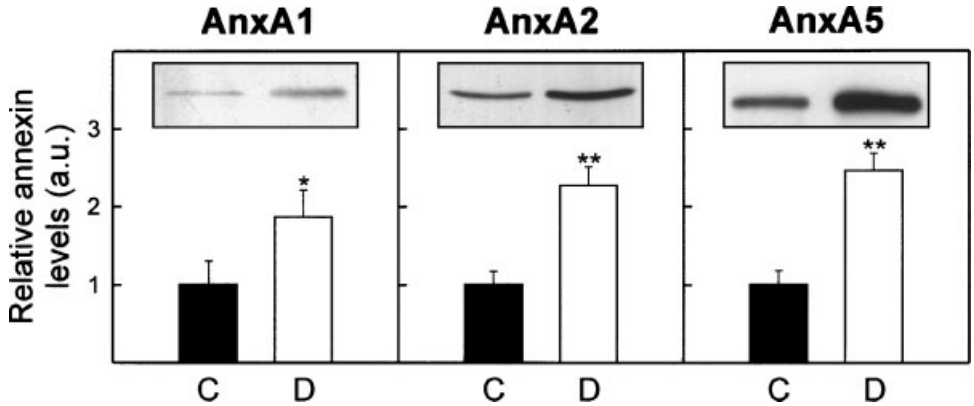

Fig. 8. Annexin expression in spontaneously differentiated Caco-2 cells. Cell lysates (15 μg protein) of Caco-2 subconfluent control cultures (C) and long-term post-confluent spontaneously differentiated cells (D) were analyzed by Western blot. In each panel a representative blot is shown. At least three different culture plates were used and analyzed independently. Data (mean ± SD) are represented in a.u. and normalized to the intensity of the band of each annexin in the corresponding control. $*P < 0.05$; $**P < 0.01$.

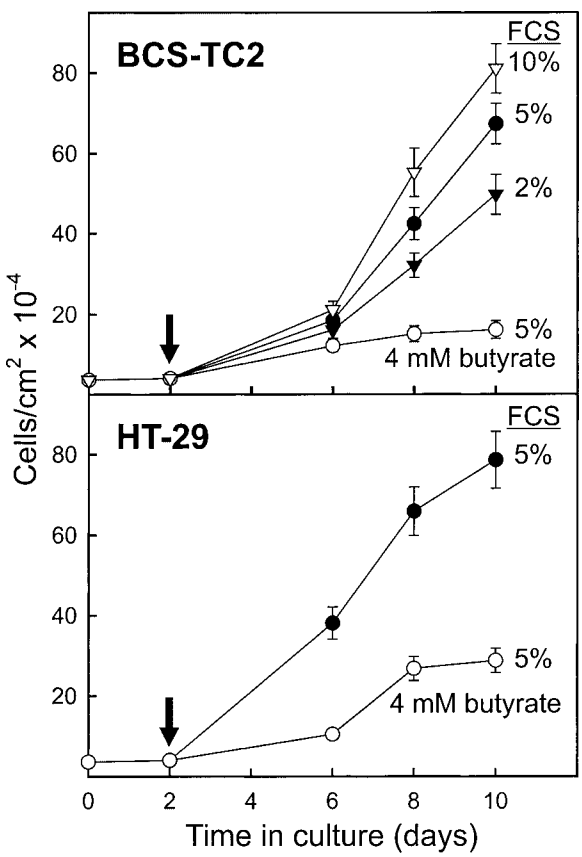

Fig. 9. Effect of butyrate treatment and serum concentration in cell proliferation. BCS-TC2 and HT-29 cells were seeded and maintained for 2 days in the presence of 5% fetal calf serum (FCS). Afterwards, medium was replaced (arrow) by fresh one containing the indicated FCS concentration, in the absence or presence of butyrate. Cell number was determined at different time points; data are the mean (±SD) of three independent experiments with duplicate samples.

low levels of AnxA1 are detected in the conditioned media of Caco-2 cells as observed in the monolayer (Fig. 1).

Butyrate treatment markedly increases the secreted annexin levels into the conditioned medium of the four cell lines. The greatest increases are achieved for secretion of AnxA1 and AnxA5, being in general smaller for AnxA2. The most significant changes are detected in BCS-TC2.2 cells, where secreted AnxA1 is increased 21-fold, AnxA5 13-fold, and AnxA2 2.4-fold.

Effect of Butyrate in Annexin Cellular Localization

Modifications in the intracellular localization of annexins after butyrate treatment were analyzed by immunofluorescence microscopy on sub-confluent cultures (Fig. 12) using DAPI counterstaining for the nuclei (not shown). Under these conditions, staining is almost identical to that obtained in confluent cultures by confocal laser microscopy (Fig. 2) but, at higher magnification, the perinuclear staining of AnxA1 and AnxA5 is more evident. Localization of AnxA1 at cell-to-cell contacts, which is detected in confluent cultures (Fig. 2), is not observed under these conditions.

We have previously described that butyrate treatment induces cell differentiation and apoptosis in BCS-TC2 cells [Navarro et al., 1997]. Differentiated cells present a significant increase in cell size showing a flatter appearance. In addition to these morphological changes, butyrate affects annexin immunostaining as well.

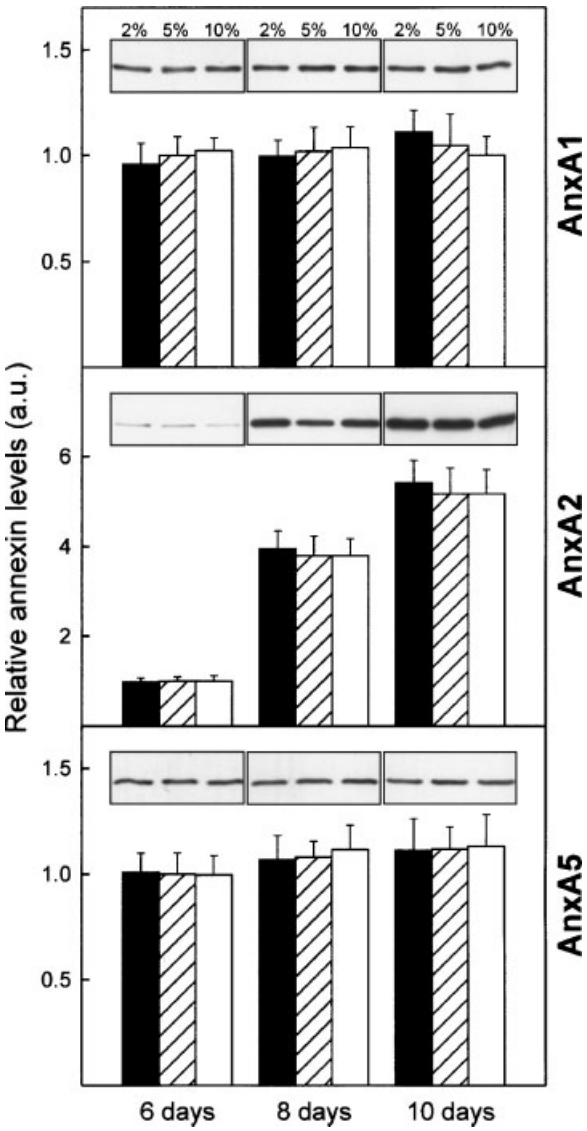

Fig. 10. Annexin expression during cell proliferation at different serum concentrations. Annexin levels were analyzed by Western blot of cell homogenates (15 μg protein) obtained after culture of BCS-TC2 cells in the presence of 2, 5, and 10% FCS, at different times. In each panel a representative blot is shown. Three independent determinations were performed for each experimental condition. Data (mean ± SD) are expressed in a.u. and normalized to the intensity of each annexin after 6 days in culture in the presence of 5% FCS. The statistical analysis shows that the only significant differences ($P < 0.01$) were found in the expression of AnxA2 with time in culture.

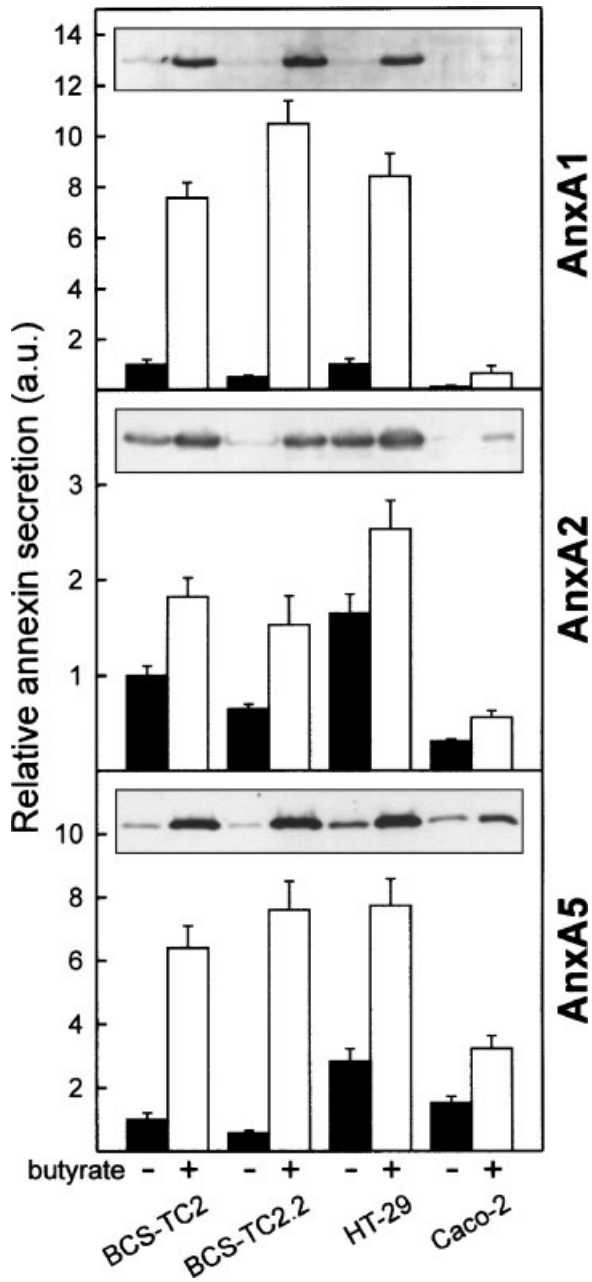

Fig. 11. Annexin secretion in control and butyrate-treated colon adenocarcinoma cells. Cells were incubated under serum-free conditions in the absence (−) or presence of 4 mM butyrate (+) for 2 days. Conditioned media were collected, concentrated, and analyzed by Western blot (20 μg). Representative blots are included in each panel. Three independent determinations were performed for each experimental condition. Data (mean ± SD) are expressed in a.u. and normalized to the intensity of each annexin in control conditioned media from BCS-TC2 cells. Differences in annexin secretion between untreated and butyrate-treated cells were significant ($P < 0.01$) in all cases.

Nevertheless, some areas of the culture still maintain the undifferentiated morphology with an annexin immunostaining pattern similar to the controls. AnxA1 and AnxA5 localization in butyrate-differentiated cells is no longer diffuse in the cytoplasm; they appear mainly associated to intracellular vesicles or membranous

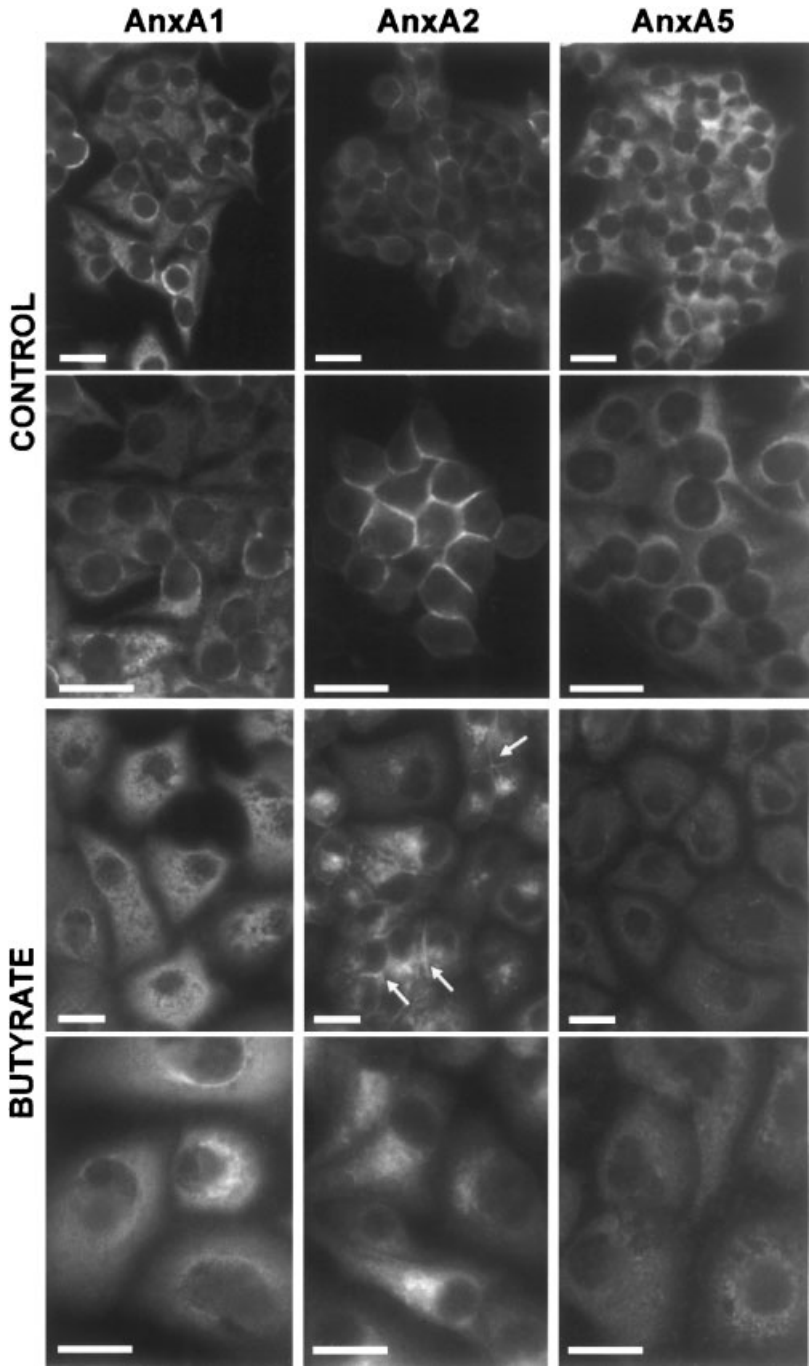

Fig. 12. Subcellular localization of AnxA1, AnxA2, and AnxA5 in control and butyrate-treated BCS-TC2 cells. Subconfluent cultures were incubated in the absence (control) or in the presence of butyrate for 4 days and then, cells were fixed and stained. Arrows indicate regions of the culture with partially differentiated cells. Scale bar = 25 μm.

structures (Fig. 12, lower panels). Counterstaining of nuclei with DAPI also reveals an apparent increase in the nuclear localization of AnxA1 and, to a lower extent, of AnxA5. The most significant changes with butyrate treatment are observed in the staining pattern of AnxA2. While in the controls (and in non-differentiated cells after butyrate treatment) AnxA2 localizes predominantly at sites of cell-to-cell contact, butyrate-differentiated cells lose the plasma membrane localization and show a cytoplasmic staining in membranous structures

close to the nucleus. Some cells in these cultures present a mixed staining in cell-to-cell contacts and intracellular membranous structures (Fig. 12, arrows).

DISCUSSION

Endogenous luminal factors present in the diet may affect the behavior of colonocytes in vivo and may be involved in colonic inflammatory processes and colorectal carcinogenesis. Butyrate, produced by fermentation of the diet fiber by the colonic flora, inhibits cell growth, promotes differentiation, and induces apoptosis in vitro [Coradini et al., 2000; Topping and Clifton, 2001; Tabuchi et al., 2002; Andoh et al., 2003] as a consequence of changes in the expression of certain genes. We have analyzed whether butyrate treatment of human colon adenocarcinoma cells could influence the expression of some annexins as these proteins are related to cell proliferation and differentiation, tumor progression, and inflammatory processes [Gerke and Moss, 2002; Perretti and Gavins, 2003; Xin et al., 2003].

Although alterations in annexin expression in different types of tumors have been described, no clear correlation has been established between these proteins and malignancy yet [Emoto et al., 2001; Gerke and Moss, 2002; Liu et al., 2003]. We have not found a relationship between the tumorigenic potential of the studied colon adenocarcinoma cells and the basal expression of these annexins. In fact, non-tumorigenic BCS-TC2 cells [Turnay et al., 1990; López-Conejo et al., 1996] show AnxA1 and AnxA5 levels almost identical to those of the tumorigenic BCS-TC2.2 or HT-29 cells; AnxA2 levels are lower, but Caco-2 cells present much lower basal amounts than the other cell lines. These results suggest a specific expression of annexins in each cell type rather than a correlation with their tumorigenicity.

An interesting correlation has been found between annexin levels and cell differentiation. We have been able to induce this process by butyrate treatment, being Caco-2 cells the ones that achieve the highest differentiation degree (Caco-2 > HT-29 > BCT-TC2 > BCS-TC2.2). In Caco-2 cells, differentiation is characterized by a butyrate-concentration dependent increase in the amount of all considered annexins. The other cell lines present a similar pattern regarding AnxA1 and AnxA5 expression changes with butyrate concentration. A significant increase in the levels of these annexins has also been found after inosine-induced differentiation and spontaneous differentiation of Caco-2 cells. These results confirm that cell differentiation of human colon adenocarcinoma cells not only increases the expression of the typical colon epithelial-cell differentiation markers (ALP, DPP-IV), but also induces an up-regulation of additional genes, among them *anxa1* and *anxa5*. Interestingly, AnxA1 has been recently reported as an effective differentiation marker in head and neck squamous cell carcinomas; well-differentiated tumors present a positive AnxA1 signal whereas poorly differentiated tumors exhibit very weak or negative staining [García Pedrero et al., 2004]. The up-regulation of these annexins with cell differentiation has also been described in other cell types: AnxA1 in lung epithelial cells [Solito et al., 1998] and keratinocytes [Sato-Matsumura et al., 2000], and AnxA5 in a human-derived glioma cell line [Giambanco et al., 1993] and in chondrocytes [Kirsch et al., 2000; Turnay et al., 2002]. Taking into account that the *anxa1* promoter presents a TATA-box whereas *anxa5* contains a TATA-less promoter [Carcedo et al., 2001], other regulatory elements in the annexin promoters must be responsible for this common behavior.

On the other hand, when AnxA2 expression is considered in butyrate-treated cells, a marked reduction in its level is observed except in Caco-2 cells. The butyrate-dependent down-regulation of AnxA2 could be due to an increased degradation or a decreased biosynthesis. One of the mechanisms described for protein level down-regulation is the stimulation of the ubiquitin–proteasome pathway, mainly described for short-life proteins such as p53 [Giuliano et al., 1999] or heat shock proteins as HSP70i [Barreto et al., 2003]. On the other hand, butyrate stimulation of the 26S proteasome, the major extralysosomal degradative machinery, has been detected in several cell lines [Giuliano et al., 1999; Yin et al., 2001]. Moreover, butyrate has been described to down-regulate the expression of wild-type and mutant p53 in colonic epithelial cells [Palmer et al., 1997]. Thus, the observed decrease in AnxA2 levels with butyrate treatment could be a consequence of proteasome activation. In fact, the treatment of BCS-TC2 cells with butyrate reduces p53 and HSP70i protein levels through the proteasome activation as the addition of

MG101 during butyrate treatment counteracts its effect on p53 and HSP70i expression. However, MG101 proteasome inhibitor does not modify the effect of butyrate on AnxA2 levels, suggesting a proteasome-independent mechanism for this down-regulation.

We have found that AnxA2 expression in BCS-TC2 cells is related to the growth state, with a significant increase in protein levels when the cells are actively proliferating, whereas AnxA1 and AnxA5 levels are not dependent on cell-growth status. A correlation between AnxA2 and cell proliferation has also been described in other systems. Its expression has been associated with proliferating areas of normal tissues or actively growing parts of primary tumors [Vishwanatha et al., 1993]. Interestingly, it has been reported that transfection of cells with an AnxA2-antisense RNA leads to inhibition of cell division [Chiang et al., 1999], what highlights the involvement of this protein in cell proliferation. Accordingly, the lower AnxA2 levels in butyrate-treated BCS-TC2 cells could be related to an impairement in cell proliferation. This negative effect on AnxA2 protein levels is quantitatively higher than the possible increase in expression upon butyrate-induced cell differentiation. In fact, growth is not impaired in inosine-differentiated cells and AnxA2 levels do not decrease, but even slightly rise, as observed in HT-29 cells. On the other hand, AnxA2 expression is enhanced by butyrate in Caco-2 cells where it induces a much higher differentiation degree. Differentiation-associated up-regulation of AnxA2 could therefore predominate in these cells over the negative effect of butyrate-induced arrest in cell growth.

Butyrate treatment not only modifies annexin levels but also induces significant changes in their subcellular localization. Non-treated cells show a predominantly uniform cytosolic AnxA1 and AnxA5 staining while AnxA2 appears close to the plasma membrane. Butyrate-induced differentiation alters annexin localization: AnxA1 and AnxA5 maintain the cytosolic staining but with a vesicle-associated pattern instead of a uniform distribution. In a similar way, AnxA2 shifts from the plasma membrane to cytosolic vesicles, although some cells still present the plasma membrane associated AnxA2. This fact could reflect the existence of cell subpopulations with heterogeneous differentiation degree. The presence of AnxA1, AnxA2, and AnxA5 in internal membranes provides additional evidence of the possible involvement of these proteins in exocytotic pathways of differentiated cells. Butyrate also induces the appearance of AnxA1 and AnxA5 nuclear staining as described for oxidative stress [Sacre and Moss, 2002] or in dependence of the growth state [Barwise and Walker, 1996] or the addition of mitogens [Kim et al., 2003].

AnxA1, AnxA2, and AnxA5 have also been located to the outer plasma membrane or secreted in several cell lines and tissues. These proteins lack a signal sequence for secretion, but it has been proposed that they may be exported to the extracellular milieu either associated to secretory vesicles [Faure et al., 2002; Gerke and Moss, 2002] or via an ATP-binding cassette transporter [Chapman et al., 2003; Wein et al., 2004]. We have detected annexin secretion in all cases, being this process enhanced by butyrate treatment. Taking into account that butyrate induces changes in the intracellular location of annexins towards a more vesicle-associated staining, it could be suggested that the increase in annexin secretion may be a consequence of exocytotic processes releasing vesicle-associated annexins to the culture medium. This process has been proposed for AnxA1 secretion in TPA-stimulated keratinocytes [Sato-Matsumura et al., 2000] and for AnxA2 secretion in chromaffin cells [Faure et al., 2002], PC12 cells after insulin receptor activation [Zhao et al., 2003], and in normal and transformed mammary epithelial cells [Kirshner et al., 2003].

The reported increase in AnxA1 expression and secretion after butyrate treatment could be an additional factor to take into account when the protective effect of butyrate and other short-chain fatty acids in different intestinal bowel diseases is considered. Butyrate not only induces the secretion of anti-inflammatory cytokines and inhibits lymphocyte proliferation and IL-2 production [Cavaglieri et al., 2003], but also induces the expression and secretion of AnxA1. This protein acts as a mediator of glucocorticoid action in inflammation probably by blocking granulocyte recruitment to the inflammation site [Walther et al., 2000] and/or via regulation of cytokine gene expression as it has been recently established in the $AnxA1^{-/-}$ mouse [Croxtall et al., 2003; Hannon et al., 2003; Yang et al., 2004]. Thus, butyrate behaves in a similar way to that described for glucocorticoids, which also increase AnxA1 secretion.

In summary, we have found a positive correlation between annexin levels and differentiation of human colon adenocarcinoma cells, but not with malignancy. AnxA2 expression is highly dependent on the cell-growth state whereas no changes are detected in AnxA1 and AnxA5. Butyrate treatment alters annexin levels and induces changes in the subcellular localization of the studied annexins towards a vesicle-associated staining that may be related to the parallel increase in annexin secretion. The increase in AnxA1 expression and secretion with butyrate may contribute to the reported anti-inflammatory effects of this agent in colon inflammatory diseases.

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