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Changes in glycosaminoglycan sulfation and protein kinase C subcellular distribution during differentiation of the human colon tumor cell line Caco-2

P. Levy, G. Cherqui, A. Robert, D. Wicek and J. Picard

Laboratoire de Biochimie, INSERM, Faculté de Médecine Saint-Antoine, 27 rue Chaligny, F-75 571 Paris Cédex 12 (France)
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Summary. During the spontaneous differentiation (day 5 to day 15 of the culture) of Caco-2 cells, the sulfation of cell layer glycosaminoglycans increased, whereas protein kinase C activity was concomitantly redistributed from the membrane to the cytosol. The protein kinase C activators, 4β-phorbol 12β-myristate, 13α-acetate and 1,2-dioctanoylglycerol inhibited glycosaminoglycan sulfation. By contrast, 4α-phorbol 12, 13 didecanoate was ineffective.
These results suggest that membrane-bound PKC may exert a modulatory effect on glycosaminoglycan sulfation, and this effect is gradually attenuated as Caco-2 cell differentiation progresses.
Key words. Differentiation; glycosaminoglycan sulfation; protein kinase C; phorbol esters; Caco-2 cells.

Glycosaminoglycans (GAGs) are complex polyanionic carbohydrates mainly associated with the cell surface and the extracellular matrix[1]. They are strategically positioned to regulate interactions between cells and their microenvironment. Such interactions are of great importance for cell growth, migration, adhesiveness and differentiation[2]. Specific alterations in cell membrane GAGs and particularly in the sulfation of their polysaccharide chains may be correlated with cellular differentiation[3]. In this regard, it was recently reported that significant changes in the sulfation of cellular GAGs took place during the differentiation of promyelocytic leukemia HL-60 cells induced by 4β-phorbol 12β-myristate, 13α-acetate (PMA)[4]. Since protein kinase C (PKC) is the primary target of PMA action[5], the enzyme was suggested to be responsible for the observed changes[4]. In the present investigation, we attempted to determine whether PKC, in the absence of exogenous activation, might play a role in the sulfation of GAGs during cellular differentiation. Therefore, we took advantage of the property of Caco-2 cells of spontaneously differentiating in vitro. This colon carcinoma cell line in the absence of any of the usual inducers of differentiation (sodium butyrate or dimethylsulfoxide), undergoes a typical enterocytic differentiation which is a growth-related phenomenon starting as soon as confluency is reached and which is characterized by: 1) morphological differentiation [presence of apical brush borders and tight junctions which are specific features of polarized epithelia[6]]; 2) functional differentiation [formation of domes and transepithelial electrical transport[7]]; these levels of differentiation are both completed by day 9[8,9]; and 3) enzymatic differentiation which is characterized by a regular increase of brush border hydrolase activities from confluency (day 6)[6,8]. Using Caco-2 cells, we examined both GAG sulfation and PKC subcellular distribution throughout cellular differentiation. In addition, we investigated the role of PKC in GAG sulfation by testing different PKC activators as well as an inactive phorbol ester known to have no effect on the enzyme.

Materials and methods

Cell culture. The Caco-2 cell line[10] was obtained from Dr. Zweibaum (Unité de Recherche sur le Métabolisme et la Différenciation des Cellules en Culture, Hôpital Paul Brousse, Villejuif, France). The cells were maintained at 37 °C in a 10% CO_2-90% O_2 air atmosphere in Dulbecco's modified Eagle medium (DMEM) (Eurobio), as previously described[8]. For experiments, cells in the 94th to 99th passage were plated at 3×10^5 cells in 25-cm^2 plastic flasks (Corning). The medium was changed 48 h after seeding and then daily in all experiments. After treatment with 0.25% trypsin[8,9], cell numbers were determined with a cell counter (Ortho Instruments).

Radiolabelling and isolation of glycosaminoglycans. At each indicated time in culture, cells were incubated with 30 μCi/ml of $Na_2{}^{35}SO_4$ (New England Nuclear, 382 Ci/mole) for 6 h; at the end of the labelling period, the radioactive medium was removed. Cell layers were successively washed six times with 5 ml 10% trichloroacetic acid for 15 min at 4 °C and twice with 5 ml ethanol. After drying, the fixed cells were dissolved in 0.5 M NaOH and neutralized with HCl. The resulting extracts were then digested at 56 °C with 1% pronase in 0.1 M Tris buffer solution plus 0.004 M $CaCl_2$ pH 8.5; after dialysis,

GAGs were isolated from digests by precipitation with ethanol and sodium acetate as previously described[11]. The uronic acid content of cell layer GAGs was estimated by the carbazole reaction modified by Bitter and Muir[12]. In experiments where the effect of PKC effectors was tested on GAG sulfation, Caco-2 cells were incubated for 1 h with $Na_2{}^{35}SO_4$ either in the absence or in the presence of the agents. The phorbol esters and 1,2-dioctanoyl-glycerol were prepared and used under the conditions described[13].

Cell fractionation and protein kinase C assay. After decanting the media and rinsing the monolayers 5 times with ice-cold phosphate buffered saline (PBS), cells were scraped free with a rubber policeman, then suspended in 2 ml ice-cold buffer A (20 mM Tris-HCl, pH 7.5 at 4 °C, 2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 50 mM 2-mercaptoethanol) and subsequently homogenized with 20 strokes of a tight teflon homogenizer. The homogenates were centrifuged for 1 h at 105000 × *g*. Supernates (cytosols) were decanted and the pellets resuspended in 1 ml of buffer A containing 0.2 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100. After 60 min at 4 °C, the solubilized membranes were centrifuged as described above. Protein content was determined by the Bradford dye method, using Bio-Rad reagent and BSA as the standard. Protein kinase C activity was determined by slight modifications of the methods previously described[14,15]. The complete reaction mixture (250 µl) contained 5 µmol Tris-HCl (pH 7.5), 2.5 µmol Mg-acetate, 25 nmoles [γ-^{32}P] ATP, 20 µg phosphatidylserine (PS), 5 µg 1,2 diolein, 0.8 mM $CaCl_2$ and 50 µg histone III-S. Basal activity (Ca^{2+}, PS-independent kinase activity) was determined by incubation of samples in the absence of PS, diolein and Ca^{2+}. Reactions were initiated by addition of the cellular extracts (10 µg of protein) and terminated after 5 min at 30 °C as in Skoglund et al.[16]. PKC activity is expressed as the activity (pmol ^{32}P transferred to histone III-S/min/10^6 cells) measured in the presence of Ca^{2+}, PS and diolein, minus the basal activity.

Results and discussion

Sulfation of cell layer glycosaminoglycans during differentiation of Caco-2 cells. To evaluate changes in cell layer GAGs during differentiation, Caco-2 cells were labelled for 6 h with $Na_2{}^{35}SO_4$ on days 5, 6, 9 and 15 of the culture. In these experiments, the radioactivity incorporated into GAGs was expressed as cpm/µg uronic acid/10^6 cells, which enabled us to evaluate the degree of sulfation of the GAGs at each time studied. As illustrated in figure 1, the specific activity of GAGs, which amounted to 2724 cpm/µg uronic acid/10^6 cells on day 5, increased markedly (by 46%) up to day 9, at which point it had reached a maximal value of 3985 cpm/µg uronic acid/10^6 cells. This specific activity of GAGs remained roughly constant till day 15 (3750 cpm/µg uronic acid/10^6 cells). These results clearly indicate that differentiation of Caco-2 cells is associated with an increase in sulfation of cell layer GAGs. This agrees with other findings that the differentiation of cell types such as HT-29 cells, another colon tumor cell line[17], as well as F9 teratocarcinoma cells[18], human monocytes[19] and myoblasts[20] has also been shown to be associated with quantitative or even qualitative modifications of GAGs, such as an increased degree of sulfation on chondroitin sulfate residues[18,19]. In the present study, we have observed that changes in GAG sulfation mainly occurred within the period day 5-day 9 of the culture. These results extend our recent observation that the structural features (sulfation pattern and hydrodynamic size) of GAGs are significantly changed when Caco-2 cells have become morphologically differentiated[9]. Considered together, our results are of particular interest in regard to the fact that these changes are concomitant with the morphological differentiation (polarization) of Caco-2 cells.

Recent information obtained on cell surface proteoglycan (PG) of mammary epithelial cells[21] have suggested that this PG covers the entire cell surface of newly cultured cells, but when the epithelial cells became polarized, cell surface PG is anchored to cytoskeleton elements by means of ionic interaction, and acts as a matrix receptor to provide the association between the epithelial skeleton with the extracellular matrix and therefore, to stabilize the morphology of epithelial sheets[21]. Thus, the PG can mediate the conversion of changes in the matrix into changes in cellular behavior such as those occurring during differentiation of the cells. In morphologically

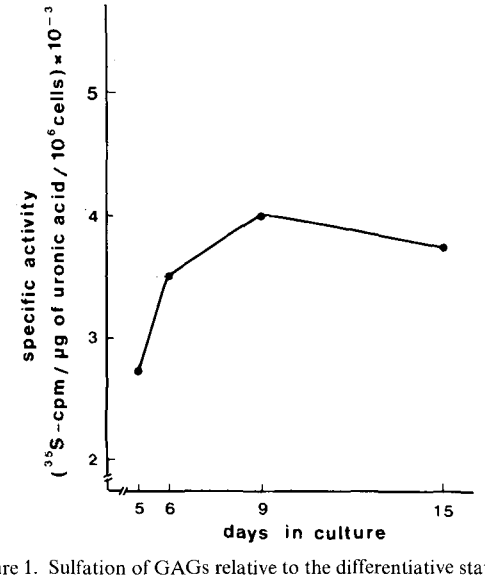

Figure 1. Sulfation of GAGs relative to the differentiative state of Caco-2 cells. Caco-2 cells were incubated for 6 h at 37 °C with $Na_2{}^{35}SO_4$ (30 µCi/ml) on days 5, 6, 9 and 15 of culture. At each indicated time, labelled proteoglycans of the cell layer were solubilized from pooled cells by alkali treatment as described under 'Materials and methods'. GAGs were liberated from proteoglycans by proteolytic digestion with pronase and then analyzed for their uronic acid (UA) content. Specific activities are expressed as the amount of radioactivity incorporated per µg UA, relative to 10^6 cells. The results are given as the means of 3 experiments performed in duplicate.

differentiated Caco-2 cells, no further modification of GAG sulfation was observed from day 9 to day 15 of the culture (at which point the enterocytic differentiation of Caco-2 cells is completed): the GAGs are immobilized by the cytoskeleton and involved in the basement membrane, to provide structural stability and support to the cells. In the present study, the observed changes in GAG sulfation during differentiation of Caco-2 cells appear to be an important step involved in the regulation of cellular functions typical of the differentiative phenotype of Caco-2 cells and may be considered as useful markers of differentiation.

Total activity and subcellular distribution of protein kinase C during differentiation of Caco-2 cells. We next investigated whether Caco-2 cell differentiation, shown to be associated with an increase in GAG sulfation, might also be associated with changes in the total activity of PKC as well as in its subcellular distribution. Towards this end, we determined the enzyme activity in both the membrane and cytosolic fractions, at various times from day 5 to day 15 of the culture. As illustrated in figure 2A, the total PKC activity of Caco-2 cells which was measured at days 6, 9 and 15, was not significantly different from that observed at day 5. By contrast, figure 2B shows that the subcellular distribution of PKC was profoundly altered within the time-course of Caco-2 cell differentiation. Thus, membrane-bound PKC, which on day 5 represented 55% of the total activity of the enzyme, progressively decreased to 14% on day 9. No further significant decrease was observed from day 9 to day 15, at which time membrane-bound PKC represented 12% of its total cellular activity. Reciprocally, cytosolic PKC activity increased from 45% on day 5 to 88% on day 15, when the cells underwent enterocyte-like differentiation. Such a preferential cytosolic localization of PKC activity in Caco-2 cells which had acquired the enterocytic phenotype is consistent with the finding that, in rabbit enterocytes, 75% of the total PKC activity was found in the cytosol[22]. On the other hand, it seems worth mentioning that

Effect of various PKC modulators on the incorporation of ^{35}S-sulfate into cell layer glycosaminoglycans of Caco-2 cells

Treatment	^{35}S-GAG specific radioactivity (% of control)
None	100
PMA (100 ng/ml	53 ± 3**
1,2 dioctanoyl-glycerol (100 µg/ml)	69 ± 5*
4 α-PDD (100 ng/ml)	93 ± 5

Confluent Caco-2 cells were incubated for 1 h at 37 °C with or without the indicated compounds in the presence of $Na_2{}^{35}SO_4$ (30 µCi/ml). Specific radioactivity of ^{35}S-GAGs was determined as indicated in figure 1. The control value (100%) was 925 ± 105 cpm/µg UA/10^6 cells. Results are given as the means ± SEM of 5 experiments performed in duplicate. Values significantly different from the control value are indicated: *$p < 0.01$, **$p < 0.001$ (as estimated by Student's t-test for non-paired data).

a quite recent study[23] also reported changes in the intracellular distribution of PKC during the PMA-induced differentiation of HL-60 cells, with the enzyme being largely cytoplasmic in the fully differentiated cells. It is therefore suggested that the alterations in PKC distribution observed in Caco-2 cells as well as in HL-60 cells are physiologically important; however, a causal relationship between PKC distribution and cellular differentiation remains to be established.

Our results clearly show that Caco-2 cells exhibit, concomitant with an increase in the sulfation of their cell layer GAGs, a decrease in membrane-bound PKC, which is the active form of the enzyme. It is noteworthy that for both processes, GAG sulfation and PKC redistribution, it is within the period day 5-day 9 that acute changes were observed whereas in the subsequent period, day 9-day 15, a stationary phase was noted. These observations suggest a relationship between sulfation of cell layer GAGs and PKC localization. It might be hypothesized that PKC, when in the membrane, negatively modulates GAG sulfation. Therefore, the increase in this latter process throughout differentiation might in fact reflect the loss of the modulation exerted by PKC, due to the redistribution of the enzyme in the cytosol.

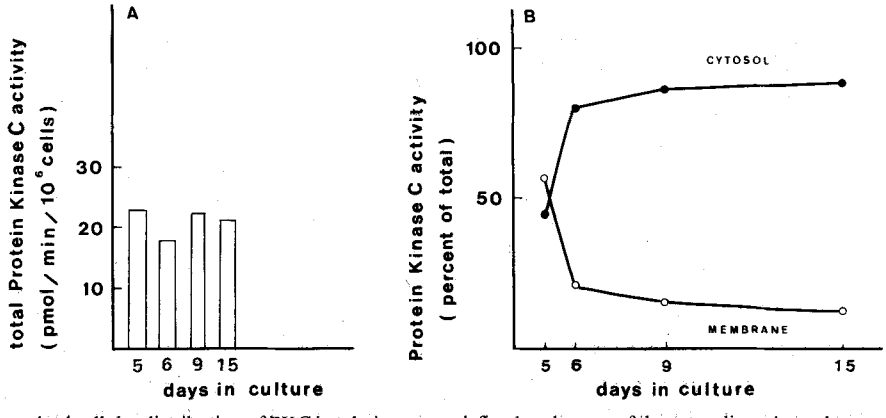

Figure 2. Total activity and subcellular distribution of PKC in relation to the differentiative state of Caco-2 cells. On the indicated days, Caco-2 cells were homogenized in 2 ml of buffer A. Cytosolic and membrane fractions were prepared and assayed for PKC activity as described in 'Materials and methods'. Panel A gives the total cellular PKC activity defined as the sum of the cytosolic and membrane activities at a given day of culture. Panel B shows the relative proportions of PKC activity found in each fraction. Each point represents the mean of 3 experiments performed in duplicate.

Effect of various PKC modulators on the sulfation of cell layer GAGs of Caco-2 cells. To test the hypothesis that activated PKC may exert a control on GAG sulfation, the effect of PMA, a phorbol ester known to activate the enzyme by promoting its translocation from the cytosol to the membrane[24], was next tested on ^{35}S-sulfate incorporation into cell layer GAGs of confluent Caco-2 cells. The table indicates that a 1-h treatment of Caco-2 cells with 100 ng/ml of PMA, a concentration shown to be maximally effective on PKC activation[24], resulted in a $47 \pm 3\%$ decrease in the sulfation of cell layer GAGs. Moreover, the table shows that 1,2-dioctanoyl-glycerol (100 µg/ml), a permeant diacylglycerol recently shown to induce in MCF-7 human breast cancer cells a discrete but significant translocation of PKC from cytosol to membrane[25], reduced GAG sulfation by $31 \pm 5\%$. By contrast, 4α-phorbol 12, 13 didecanoate (4α-PDD, 100 ng/ml), an inactive phorbol ester, proved ineffective in modifying this process (table); this provides an assessment of the specificity of the effect of PMA. Our finding that PKC activators decreased GAG sulfation whereas an inactive phorbol ester was without effect on this process is consistent with the results reported elsewhere[4, 26]. Such a finding further supports the hypothesis that the enzyme, once activated, may exert a negative control on GAG sulfation.

In conclusion, our results provide evidence that the spontaneous differentiation of Caco-2 cells is associated with an increase in the sulfation of cell layer GAG which is concomitant with a redistribution of PKC from the membrane to the cytosol. These data, together with the finding that PKC activators reduce GAG sulfation, suggest that in non-differentiated Caco-2 cells which are characterized by the highest level of membrane-bound PKC, the enzyme exerts a negative modulation on this process; this effect may be gradually lost as PKC activity redistributes to the cytosol over the time-course of spontaneous differentiation.

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Thyroxine-induced redistribution of creatine kinase isoenzymes in rat cardiomyocyte cultures

H. Brik, L. Alkaslassi, D. Harell[a], O. Sperling[a] and A. Shainberg

Bar-Ilan University, Department of Life Sciences, Ramat-Gan 52100 (Israel), and [a] Department of Chemical Pathology, Beilinson Medical Center, Petah-Tikva (Israel)
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Summary. The present study demonstrates a change occurring in the creatine-kinase isoenzyme profile of cardiomyocyte cultures induced by a chronic administration of excessive amounts of thyroid hormones (TH). This change is manifested by an increased level of the CK-BB isoenzyme, generally at the expense of CK-MM isoenzyme. The elevation of CK-BB is probably a result of a specific effect of TH through activation of gene expression, rather than a contribution of an increased number of non-myocardial cells. The implications of these results in the diagnosis of heart failures are discussed.
Key words. Creatine-kinase isoenzymes; cardiomyocyte cultures; thyroid hormones.