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Original article

Evidence for a modulatory role of protein kinase C on glycosaminoglycan biosynthesis during the spontaneous differentiation of Caco-2 cells

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Summary — The role of protein kinase C (PKC) in the regulation of glycosaminoglycan (GAG) sulfation was investigated during the spontaneous differentiation of Caco-2 cells. The total cellular activity of PKC as well as its subcellular distribution was examined from d 5 (non-differentiated cells) to d 15 (enterocytic differentiated cells): during this period, PKC was redistributed from the membrane to the cytosol, but the amount of PKC activity was not modified. This redistribution of PKC was concomitant with an increase in ^{35}S-sulfate incorporation in GAG. 4-β phorbol 12 β-myristate, 13-α acetate (PMA) and 1-2 dioctanoyl-glycerol (DIC8), 2 PKC activators, decreased ^{35}S-sulfate incorporation in GAG; by contrast, 4α-phorbol 12,13 didecanoate (4α-PDD), an inactive phorbol ester, proved to be ineffective. These results suggest that membrane-bound PKC which is the active form of the enzyme, may exert on GAG sulfation a modulatory role, which is gradually attenuated as Caco-2 cell differentiation progresses.

differentiation / glycosaminoglycan sulfation / protein kinase C / phorbol esters / Caco-2 cells

Résumé — Rôle modulateur de la protéine kinase C sur la biosynthèse des glycosaminoglycannes au cours de la différenciation spontanée des cellules Caco-2. *Nous avons étudié le rôle de la protéine kinase C (PKC) dans la régulation de la sulfatation des glycosaminoglycannes (GAGs) au cours de la différenciation des cellules Caco-2. L'activité cellulaire totale de la PKC de même que sa distribution subcellulaire ont été examinées dans l'intervalle s'écoulant du 5^{e} j (cellules non différenciées) au 15^{e} j (cellules différenciées) du temps de culture. Pendant cette période, l'activité cellulaire totale de la PKC n'est pas modifiée alors que l'enzyme est redistribuée à partir de la membrane vers le cytosol. Cette redistribution s'accompagne d'une augmentation du degré de sulfatation des GAGs de la couche cellulaire. Le phorbol myristate acétate (PMA) et le 1,2-dioctanoylglycérol (DIC8), 2 activateurs de la PKC, diminuent la sulfatation des GAGs tandis que le phorbol didécanoate (4α-PDD) est inefficace. L'ensemble de nos résultats suggère que la PKC, quand elle est sous sa forme membranaire, c'est-à-dire active, pourrait exercer sur la sulfatation des GAGs un rôle modulateur qui s'atténue au fur et à mesure que la différenciation progresse.*

différenciation / sulfatation des glycosaminoglycannes / protéine kinase C / ester de phorbol / cellules Caco-2

INTRODUCTION

Cell surface glycosaminoglycans are complex polyanionic carbohydrates which have been shown to undergo specific alterations during the time course of cellular differentiation (Kolset and Kjellen, 1986). Significant changes in the sulfation of cellular GAGs were found to take place during the differentiation of promyelocytic leukemia HL-60 cells induced by PMA (Carson and Baxter, 1986). Since PKC is the primary target of PMA action (Castagna *et al,* 1982), the enzyme was suggested to be responsible for the observed changes. 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 to spontaneously differentiate *in vitro.* Indeed, this colon carcinoma cell line undergoes, in the absence of usual inducers of differentiation, a typical enterocytic differentiation characterized by a polarization of the cells with the presence of tight junctions, apical brush borders and the associated hydrolases (Rousset *et al,* 1985). Using this cell line, 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

Characteristics of Caco-2 cell differentiation

The Caco-2 cell line (Fogh *et al,* 1977) was obtained from Dr Zweibaum (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) (Pinto *et al,* 1983; Lévy *et al,* 1988). On d 5 after plating, cells were in a non-differentiated state (Pinto *et al,* 1983; Rousset *et al,* 1985). Enterocytic differentiation is a growth-related phenomenon and is characterized by: 1) morphological differentiation (presence of apical brush borders and tight junctions which are specific features of polarized epithelia (Rousset *et al,* 1985); 2) functional differentiation (formation of domes and transepithelial electrical transport (Grasset *et al,* 1984); these levels of differentiation are both completed by d 9 (Pinto *et al,* 1983; Lévy *et al,* 1988); and 3) enzymatic differentiation which is characterized by a regular increase of brush border hydrolase activities from confluency (d 6) (Pinto *et al,* 1983; Rousset *et al,* 1985).

Radiolabelling and isolation of glycosaminoglycans

Caco-2 cells were labelled with 30 μCi/ml of $Na_2{}^{35}SO_4$ (New England Nuclear, 382 Ci/mol) on d 5, 6, 9 and 15 of the culture. After a 6 h incubation period, the radioactive medium was removed. Cell layers were successively washed with trichloroacetic acid for 15 min at 4 °C and with ethanol. After drying, the fixed cells were dissolved in 0.5 M NaOH and neutralized with HCl. GAGs were isolated by digestion with pronase (Lévy *et al,* 1984). The uronic acid content of cell layer GAGs was estimated by the carbazole reaction modified by Bitter and Muir (1962). 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 phorbol esters as previously described (Cherqui *et al,* 1986). The radioactivity incorporated into GAGs was expressed as cpm/μg uronic acid/10^6 cells, which enabled us to evaluate the sulfation of GAGs at each time studied.

Cell fractionation and protein kinase C assay

Cells were scraped free with a rubber policeman, then suspended in ice-cold buffer A (20

mmol/l Tris-HCl, pH 7.5 at 4 °C, 2 mmol/l EDTA, 5 mmol/l EGTA, 0.25 mol/l sucrose, 50 mmol/l 2-mercaptoethanol) and subsequently homogenized with a tight teflon homogenizer. The homogenates were centrifuged for 1 h at 105 000 g. Supernates (cytosols) were decanted and the pellets resuspended in 1 ml of buffer A containing 0.2 mmol/l 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 and protein kinase C activity was determined as described in Cherqui *et al* (1989).

Statistical analysis

Results are given as means ± SEM for the indicated numbers of experiments with separate preparations of cells. Differences between the mean values were evaluated with the non-paired Student's *t* test.

RESULTS AND DISCUSSION

Glycosaminoglycan sulfation during differentiation of Caco-2 cells

As shown in figure 1, the sulfation of GAGs markedly increased by 46% from d 5 to d 9; by contrast, no further increase in this sulfation was observed between d 9 and d 15. 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 (Simon-Assman *et al,* 1987), as well as human monocytes (Kolset *et al,* 1983) and myoblasts (Hutchinson and Yansin, 1986) has also been shown to be associated with quantitative or even qualitative modifications of GAGs. In the present study, we have observed that changes in GAG sulfation mainly occurred within the period d 5–d 9 of the culture. These results extend our recent observation that the structural features of GAGs are significantly changed when Caco-2 cells become morphologically differentiated (Lévy *et al,* 1988). Combined, our results are of particular interest with regard to the fact that these changes are concomitant with the morphological differentiation (polarization) of Caco-2 cells.

Changes in the localization of protein kinase C during differentiation of Caco-2 cells

The total PKC activity of Caco-2 cells was not appreciably modified during the period

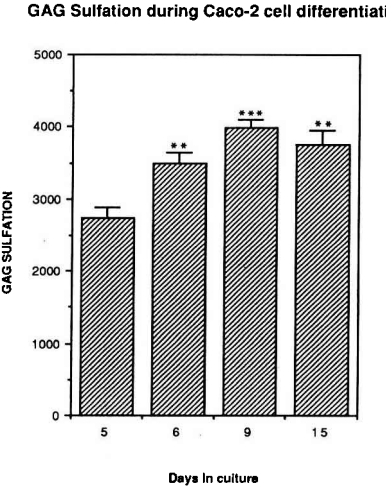

Fig 1. Sulfation of ^{35}S-labelled glycosaminoglycans during differentiation of Caco-2 cells. At each indicated time of culture, Caco-2 cells were incubated for 6 h with $Na_2{}^{35}SO_4$ (30 μCi/ml). After solubilization of labelled proteoglycans of the cell layer by alkali treatment, GAGs were isolated by proteolytic digestion with pronase and then analyzed for their uronic acid (UA) content. The results are given as the means ± SEM of 3 experiments performed in duplicate. Values significantly different from that obtained in non-differentiated cells (d 5) are indicated: ** $P < 0.02$, *** $P < 0.01$.

d 5–d 15, whereas the subcellular distribution of PKC was profoundly altered (fig 2). Membrane-bound PKC, which on d 5 represented 55 ± 6% of the total activity of the enzyme, progressively decreased to 14 ± 5% on d 9. No further significant decrease was observed from d 9 to d 15, at which time membrane-bound PKC represented 12 ± 4% of its total cellular activity. Reciprocally, cytosolic PKC activity increased from 45 ± 6% on d 5 to 88 ± 4% on d 15, when the cells underwent enterocyte-like differentiation (fig 2). In the same line, a previous study 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 (Kiss *et al*, 1988). These changes observed in HL-60 cells as well as in Caco-2 cells may have biological significance. Since the latter differentiate into enterocytes, a cell type where membrane transport is an important physiological activity, the distribution of PKC should play a crucial role in this activity.

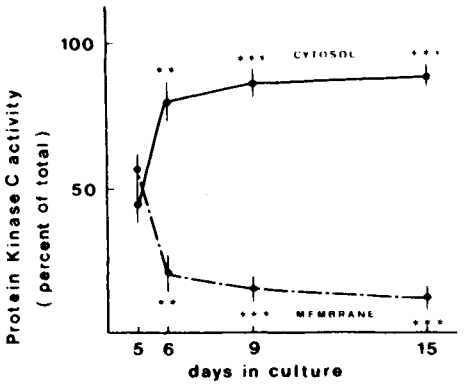

Fig 2. Subcellular distribution of protein kinase C during differentiation of Caco-2 cells. Cytosolic and membrane fractions were prepared and assayed for PKC activity as described in *Materials and Methods.* Results are expressed as the relative proportions of the total PKC activity found in cytosolic and membrane fractions. Total PKC activity amounted to 23.1 ± 1.6, 18.2 ± 1.3, 22.6 ± 1.6 and 21.3 ± 1.2 pmol ^{32}P respectively transferred to Histone III-S/min/mg protein on d 5, 6, 9 and 15 of the culture. Each point represents the mean ± SEM of 3 experiments. Values significantly different from that obtained in non-differentiated cells (d 5) are indicated: ** $P < 0.02$, *** $P < 0.01$.

From the above results, it is clear that Caco-2 cells exhibit, concomitant with an increase in the sulfation of their cell layer GAGs, a decrease in membrane-bound PKC, *ie* the active form of the enzyme. Of particular interest is the fact that for both processes, -GAG sulfation and PKC redistribution-, it is within the period d 5–d 9 that acute changes were observed whereas in the subsequent period, d 9–d 15, a stationary phase was noted. These observations which suggest a relationship between sulfation of cell layer GAGs and PKC localization, lead us to propose that PKC, when in the membrane, negatively modulates GAG sulfation.

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

Treatment	*^{35}S-GAG sulfation (% of control)*	
None	100	
PMA (100 ng/ml)	53 ± 3	($P < 0.001$)
DIC 8 (100 μg/ml)	69 ± 5	($P < 0.01$)
4 α-PDD (100 ng/ml)	93 ± 5	

Effect of various PKC modulators on the sulfation of cell layer GAGs of Caco-2 cells

Table I indicates that a 1 h-treatment of Caco-2 cells with 100 ng/ml of PMA, a phorbol ester known to activate the enzyme by promoting its translocation from the cytosol to the membrane (Kraft and Anderson, 1983), resulted in a 47 ± 3% decrease in the sulfation of cell layer GAGs. In addition, DIC8 (100 μg/ml) a permeant diacylglycerol, reduced GAG sulfation by 31 ± 5% (table I). By contrast, 4α-PDD (100 ng/ml), an inactive phorbol ester, proved ineffective in modifying this process (table I) which provides an assessment of the specificity of the effect of PMA. These findings which are consistent with previous data (Carson and Baxter, 1986; Hasumi *et al*, 1987) lend support to the hypothesis that the enzyme, once activated, negatively modulates GAG sulfation.

CONCLUSION

Our results show that during the spontaneous differentiation of Caco-2 cells, PKC undergoes a redistribution from the membrane to the cytosol which is concomitant with an increase in the sulfation of cell layer GAGs. Moreover, PKC activators which promote the translocation of the enzyme from the cytosol to the membrane, decrease GAG sulfation. Combined, these findings suggest that in non-differentiated Caco-2 cells where membrane-bound PKC is the predominant form of the enzyme, PKC negatively controls the sulfation of cell layer GAGs. This negative modulation exerted by the active form of the enzyme may gradually decline together with the redistribution of PKC in the cytoplasm over the time-course of the differentiation of Caco-2 cells.

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