Blocking of lectin-like adhesion molecules on pulmonary cells inhibits lung sarcoma L-1 colonization in BALB/c-mice

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Summary. Adhesion and inhibition experiments with pulmonary cells of BALB/c-mouse origin and syngeneic sarcoma L-1 cells indicated that L-fucose specific lectin-like adhesion molecules, presumably situated on pulmonary cell surfaces are (at least partly) responsible for the specificity of this cell-cell interaction. Addition of specific sugars and glycoconjugates (L-fucose and fucoidan, respectively) to the incubation medium evidently inhibited the adhesion process as quantified using radiolabelled tumor cells. Unspecific carbohydrates (e.g. D-galactose) did not affect the cellular interaction. In vivo, repeated administration of fucoidan (but not of unspecific glycoconjugates) significantly inhibited the settling of metastatic sarcoma L-1 cells in the lungs of BALB/c-mice. Therefore, when lectin-like adhesion molecules on pulmonary cells were blocked with competitive glycoconjugates, tumor cell colonization of the lung could be significantly inhibited.

Keywords. Glycoprotein; sarcoma L-1 tumor cells; L-fucose; fucoidan; lung metastasis; BALB/c-mice.

Studies of neoplastic spread in man, as well as in experimental animal models, have revealed that it is a complex process consisting of numerous sequential steps[1,2]. In many tumor systems (e.g. sarcoma L-1/BALB/c-mouse) lung and liver are the organs most frequently involved in the formation of secondary tumor colonies. In the course of our investigations we found that organ-characteristic lectins (e.g. the hepatic lectin, discovered by Ashwell and Morell[3]), can apparently act as an acceptor of neoplastic cells by interacting with specific carbohydrate receptors on the surface of metastatic tumor cells. In view of the galactosyl specificity of the hepatic lectin we have suggested and demonstrated that administration of competitive carbohydrate-bearing glycoconjugates (e.g. arabinogalactan, D-galactose) significantly inhibited the metastatic spread of tumor cells into the livers of experimental mice[4].

Recently, a fucose binding protein with the characteristics of a lectin was detected on the surface of pulmonary cells[5] and it was speculated that it might be involved in metastatic lung colonization. Indeed, a positive correlation has been found between elevated tumor cell fucose content and tumorigenicity and metastasis in many experimental tumor models[6–8]. Similarly, patients with progressively growing and metastasizing tumor cells have been found to have elevated serum fucose levels[9,10] and fucosyltransferase activities[11] presumably as a result of tumor cell surface shedding and/or altered secretion by host cells[12]. Recent studies on the synthesis and expression of cell surface carbohydrates in sarcoma L-1 cells revealed considerable amounts of fucose, beside other sugar components. Gas-liquid-chromatographic examinations indicated that inhibition of the cellular glycoprocessing by tunicamycin, swainsonine and related substances greatly reduced detectable fucose and other carbohydrates on sarcoma L-1 cells and significantly inhibited pulmonary colonization in BALB/c-mice[13].

In this study we investigated the receptor-mediated adhesion process of sarcoma L-1 tumor cells and pulmonary cells and its specific inhibition by receptor analogues in vitro. In vivo (BALB/c-mice) lung tumor colonization was examined and quantified after blockade of lectin-like adhesion molecules on pulmonary cells with competitive sugars or glycoconjugates.

Materials and methods

Animals. Inbred male BALB/c-mice (Zentralinstitut für Versuchstiere GmbH, Hannover, FRG), 8–12 weeks old, weighing 20–22 g, were used for the in vivo studies. The animals were kept in plastic cages and allowed free access to food and water.

Tumor. For all experiments sarcoma L-1 tumor (Institute of Oncology, Warsaw, Poland) was selected. This tumor arose spontaneously in the lung of a BALB/c-mouse and was maintained in this strain. Material was used from serial passages (120–130) of the tumor. Primary tumors were dissected from donor mice, minced with scissors and passed through a steel sieve. The cells were washed, suspended in RPMI-1640 (Gibco Co. Grand Island, N.Y., USA) and counted. All cell suspensions injected into mice were > 95% viable as assessed by trypan blue dye exclusion, and were examined microscopically for signs of aggregation. Suspensions exhibiting any obvious aggregates were discarded since the extent of tumor cell clumping can affect colonization capacity[14]. Preparation and administration of the cells were as previously described[4].

Labelling of tumor cells. Sarcoma L-1 cells (2×10^5/ml) were cultivated in RPMI-1640 (Gibco Co.) supplemented with 10% fetal calf serum (FCS) and 2 μCi of ^{3}H-thymidine (Amersham, Braunschweig, FRG) for 24 h. Cells were washed and suspended in RPMI-1640, and the radioactivity of the sample was checked.

Pulmonary cells. According to the method of Douglas and Kaighn[15], suspensions of lung cells from BALB/c-mice were obtained by dissociation and enzymatic digestion (collagenase, pronase, both obtained from Sigma Chemicals Co. St. Louis, MO., USA). The suspension was cultured in petri dishes in RPMI-1640 (Gibco Co.) supplemented with 10% FCS, 2 mM L-glutamine, 100 units of penicillin/ml and 100 µg of streptomycin/ml. The cells were maintained at 37 °C in a humified incubator with 5% CO_2. The medium was replaced three times a week.

Experiments. 1×10^6 ^{3}H-thymidine labelled sarcoma L-1 cells were added to microplates with growing lung cells. After 2 h of incubation (partly in glycoconjugate-containing medium) non-adherent cells were gently removed by repeated washing with RPMI-1640 before versene (1 ml; 1:5000; Sigma Chemicals Co.) was added. After 30 min pulmonary cells and adherent tumor cells were harvested and radioactivity was measured in a liquid scintillation counter (Beckmann Ltd. München, FRG). All glycoconjugates (fucoidan, L-fucose, D-galactose, Sigma Chemicals Co.) were added to the medium at concentrations of 2 mg/ml and were present during the whole period of incubation. Furthermore, all glycoconjugates were tested for their capacity to induce homotypic adhesion of sarcoma L-1 cells[14] but no evidence for such an ability was found. For documentation (microphotograph) and further in vivo investigations these experiments were performed without ^{3}H-thymidine labelling of the tumor cells. To check their metastatic capicity, 1×10^4 of the non-adherent sarcoma L-1 cells were i.v. inoculated into BALB/c-mice and the pulmonary colonization was compared with that of the heterogenous tumor population as described in the following section.

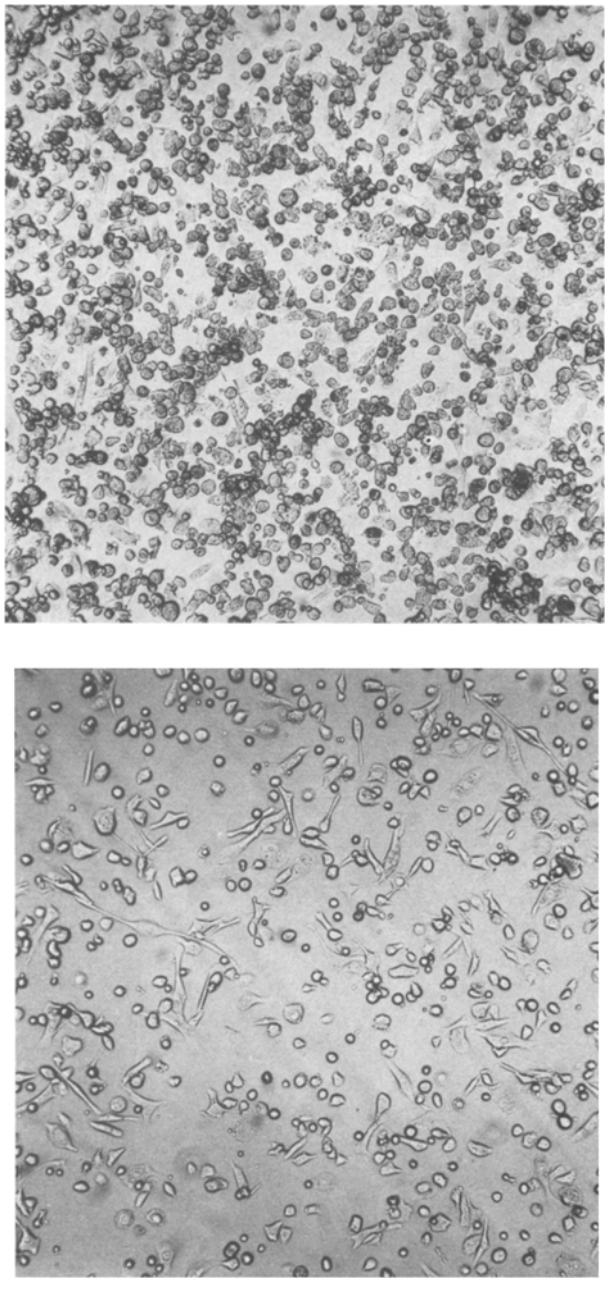
For the in vivo studies, sarcoma L-1 cells (1×10^4 and 5×10^4) were i.v. inoculated into the tail veins of syngeneic BALB/c-mice (10 mice for each group). Fucoidan (a polysaccharide of sulfated L-fucose) or D-galactose (1 mg and 2.5 mg per 0.1 ml) or heparin (Sigma Chemicals Co., 1 mg per 0.1 ml) were solubilized in PBS, i.p. pre-injected (1 h before tumor cell inoculation) and regularly administered for 4 days (24-h intervals). Preliminary investigations had shown that these doses of glycoconjugates as well as the schedule of administration and tumor cell dose were optimal. Furthermore, no side effects of this regimen were obvious in mice. Lung surface tumor nodules were counted 14 days after sarcoma L-1 inoculation according to the method of Hill and Bush[16].

The anticoagulant activity of heparin and fucoidan was determined using BALB/c-mouse plasma in the activated partial thromboplastin time (PTT) and thrombin time (TT) tests as recently proposed[17–19]. The animals were treated i.p. for 4 days with heparin or fucoidan (1 mg per mouse and 24 h). To obtain plasma (2–4 h after final treatment), blood was collected by cardiac puncture of anaesthetized mice, 9 volumes of mouse blood being drawn into one volume of 3.8% sodium citrate. The erythrocytes were removed by centrifugation (10,000 × g) and the plasma was tested for anticoagulant activity.

Statistics. Significance of data (p) was calculated by Student's t-test. All experiments were performed in triplicate.

Results

Adhesion studies with pulmonary cell monolayers of BALB/c-mouse origin were performed with syngeneic sarcoma L-1 cells. As shown in the figure a spontaneous adhesion occurred in carbohydrate-free culture medium



Microphotograph of pulmonary cell monolayer and sarcoma L-1 cells adhering to the pulmonary cells (maintained in primary cultures) after 2-h incubation at 37 °C (a). Addition of specific glycoconjugate (fucoidan) to the incubation medium substantially inhibited adhesion (b). Unspecific carbohydrates (D-galactose) did not affect cellular interaction.

independent of the metabolic activities of the cells. The mechanism of sarcoma L-1/pulmonary cell recognition was assessed using glycoconjugates in order to determine whether this type of recognition was sugar-dependent, as described elsewhere [5]. Accordingly, cell adhesion assays were performed in the presence of glycoconjugates as putative inhibitors. The figure shows that addition of fucoidan (a polysaccharide of sulfated L-fucose) to the incubation medium substantially inhibited the adhesion of sarcoma L-1 cells to pulmonary cells. Comparable results could be achieved after addition of L-fucose to the incubation medium. However, addition of unspecific carbohydrates (D-galactose) did not affect the adhesion process. Using radiolabelled sarcoma L-1 cells the adhesion efficiency could be quantified. The results are summarized in table 1 and indicated a significantly reduced cellular adhesion (expressed as reduced counts per minute, cpm) after addition of fucoidan to the incubation medium. L-fucose as well was found to be a potent inhibitor (though the reduction was non-significant), whereas unspecific carbohydrates (D-galactose) did not reduce the adhesion process.

To check the effect of sulfated polysaccharides on the anticoagulant activity in BALB/c-mice, partial thromboplastin time (PTT) and thrombin time (TT) were measured after heparin and fucoidan treatment, respectively. As compared to untreated control mice (PTT = 25 ± 2 s, TT = 18 ± 3 s) both fucoidan and heparin evidently had similar effects on the coagulation state of recipient mice (PTT = >120 s, TT = >60 s).

After establishing the most favorable glycoconjugate dosage and timing scheme in preliminary investigations, the BALB/c-mouse – sarcoma L-1 model system was selected for the in vivo studies. This model system proved to be optimal since i.v. inoculation of the tumor cells exclusively induced lung colonization. The appearance of tumor colonies in organs other than lung has never been observed in this model system, unless sarcoma L-1 cells were pretreated with neuraminidase [4]. Pre-injection (1 h before i.v. inoculation of the tumor cells) and regular i.p. re-injection of fucoidan (for 4 days in 24-h intervals) significantly inhibited the settling of sarcoma L-1 tumor nodules into the lungs of syngeneic mice. Unspecific carbohydrates (e.g. D-galactose) and sulfated polysaccharides (e.g. heparin) administered in equivalent dosage and timing scheme did not affect the number of pulmonary tumor nodules (table 2).

Additional experiments were performed to roughly assess the metastatic capacity of sarcoma L-1 cell subpopulations and the specificity of the metastatic process. Accordingly, the number of pulmonary tumor nodules after i.v. inoculation of 1×10^4 sarcoma L-1 cells, or of the non-adherent population taken from tissue cultures after performing the in vitro adhesion experiments, were compared. As expected, injection of the non-adherent sarcoma L-1 population clearly induced fewer pulmonary tumor nodules (1.75 ± 0.8) as compared to the whole population (9.75 ± 1.3). Statistically, this difference was significant ($p < 0.01$), and it suggests that the metastatic process is very specific.

Table 1. Inhibitory effect of glycoconjugates on adhesion of ^{3}H-thymidine labelled sarcoma L-1 cells to pulmonary cells of BALB/c-mouse origin

Substance added to the incubation medium (2 mg/ml)	Adherence of labelled L-1 cells to pulmonary cells in vitro			
	cpm	±SD	%	p
Control	2798	319	100	–
Fucoidan	899	503	32.1	<0.05
L-fucose	1416	408	50.6	n.s.
D-galactose	2933	450	104.8	n.s.

Experiments were performed in triplicate; p values < 0.05 are significant; n.s. = not significant.

Table 2. Mean number of lung colonies in mice injected with tumor cells and treated with glycoconjugate

Treatment of groups of 10 BALB/c-mice each with:	Mean number of lung colonies (± SD) after inoculation of L-1 cells	
	$1 \cdot 10^4$	$5 \cdot 10^4$
PBS (control)	8.6 (±0.5)	150 (±17.6)
Fucoidan 2.5 mg	0.2 (±0.4)*	20.7 (±12.4)**
Fucoidan 1 mg	n.t.	34.3 (±14.4)**
D-galactose 2.5 mg	9.0 (±1.4)	159 (±25.5)
Heparin 1 mg	9.2 (±1.8)	140 (±36.5)

Fucoidan (2.5 and 1 mg), D-galactose (2.5 mg) and heparin (1 mg) were i.p. administered to groups of 10 BALB/c-mice each (1 h before tumor cell inoculation and for 4 days in 24-h intervals). *$p < 0.001$, **$p < 0.01$; both are significantly different from controls; PBS = phosphate buffered saline; n.t. = not tested.

Discussion

Recent studies on the nature of metastasis have shown that only a small subpopulation of cells in tumors possess the necessary characteristics for their release from the primary tumor and transport to and establishment of tumor foci in distant organs [1, 20]. Carbohydrate moieties on cell surface glycoconjugates were suggested to play an important role in the metastatic spread [13, 21] since it could be demonstrated that they are involved in adhesion processes [5, 22]. Cell surface carbohydrates may thus be recognized by membrane glycosyltransferases [23], membrane glycosidases [24] or by membrane lectins [25] which are sugar-binding proteins of non-immune origin that can induce cell agglutination or glycoconjugate precipitation [26]. Based on previous data from different experimental tumor systems which suggested that the metastatic dissemination of tumor cells into the liver can be inhibited by tolerable concentrations of receptor-complementary glycoconjugate which block the organ-characteristic lectins [4, 27, 28], we initiated studies on pulmonary cells and syngeneic tumor cells, both of BALB/c-mouse origin. Recent investigations of Kieda and Monsigny manifested a fucose-binding protein on the surface of pulmonary cells which apparently played a role in tumor cell adhesion in preliminary experiments [5]. These findings are in accordance with positive correlations which have been found between elevated tumor cell fucose content and tumorigenicity and metastasis in various tumor mod-

els[6-8]. Furthermore, sarcoma L-1 cells could be shown to contain high amounts of L-fucose (beside other carbohydrates) which obviously influence their metastatic capacity[13].

The experiments reported here show that in vitro a spontaneous adhesion occurs after co-incubation of BALB/c-mouse pulmonary cells and sarcoma L-1 cells. Addition of specific carbohydrate (L-fucose, fucoidan) to the incubation medium significantly inhibited the adhesion process whereas unspecific glycoconjugates did not affect the cell-cell interaction. Since the binding of tumor cells by parenchymal cells is not a non-specific aggregation of cells but the result of a specific recognition process, pulmonary cells may therefore form a privileged target for certain (L-fucose containing) metastatic cells in vivo. Recent experiments are in accordance with this speculation and show that the 'homing' of sarcoma L-1 cells into the lung can be significantly decreased by temporary administration of receptor-complementary glycoconjugates. This lectin-mediated recognition process is highly specific[2,28] and apparently related to the steric arrangement of the specific glycoconjugate (fucoidan, L-fucose) fitting into the combining site of the lectin, as could be demonstrated for the galactosyl-specific hepatic lectin previously[4,28]. The most appropriate carbohydrate-bearing molecules for in vivo application would be those which are non-toxic, have a long in vivo half-life, and express the right carbohydrates in the correct steric arrangement, and a density sufficient for multiple ligand interactions with the corresponding cell surface lectins of organ parenchymal cells. It is clear that the glycoconjugates used in this study are not optimal with respect to all these criteria, and that more research into such ligands is required. However, their use can also be considered as a possibility for elucidating basic mechanisms of metastatic tumor spread, and should be distinguished from the immunomodulating potency of sulfated polysaccharides[29], since this effect requires triggering of the immune system before challenge[30].

Although recent experiments from our laboratories demonstrated that heparin had no effect on the arrest of sarcoma L-1 cells in syngeneic BALB/c-mice[31], the effect of sulfated polysaccharides (heparin and fucoidan) on the coagulation state of BALB/c-mice was evaluated. Accordingly, partial thromboplastin time (PTT) and thrombin time (TT) were measured since anticoagulants were believed to decrease the number of metastatic lesions, at least in certain tumor models[17,18]. In accordance with recent studies of Coombe et al.[19], both heparin and fucoidan exhibited comparable end-points in the anticoagulant assays after 4 days of treatment; these were evidently prolonged as compared to non-treated control animals. These data support the previously stated view[18,19] that (analogous to other tumor models) in the case of the sarcoma L-1/BALB/c-mouse model the anticoagulant activity of sulfated polysaccharides apparently plays a minor role in their antimetastatic action. This conclusion was based on the observation that heparin treatment did not affect the pulmonary sarcoma L-1 colonization of BALB/c-mice, whereas comparable fucoidan injections significantly reduced the tumor load in the lungs. The displacement of tumor cells from the lung observed after fucoidan treatment is thus not due simply to the administration of sulfated polysaccharide, but appears to be specific. The recent finding that a negative correlation exists between sulfated polysaccharides that were bound to the surface of certain tumor cells and those that inhibited metastasis[19] favors the assumption that specific polysaccharides may interfere with adhesion molecules on endothelial or pulmonary cells in a lectin-like manner.

In conclusion, membrane lectins and membrane glycoconjugates are able to mediate a specific binding to cells which display complementary membrane glycoconjugates and membrane lectins respectively[27,32]. This might have therapeutic possibilities in the future.

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1 Fidler, I. J., Breast Cancer Res. Treatment *9* (1987) 17.
2 Schirrmacher, V., Altevogt, P., Fogel, M., Dennis, J., Waller, C. A., Barz, D., Schwartz, R., Cheingsong-Popov, R., Springer, G., Robinson, P. J., Nebe, T., Brossmer, W., Vlodavsky, J., Pawelek, N., Zimmermann, H. P., and Uhlenbruck, G., Invasion Metastasis *2* (1982) 313.
3 Ashwell, G., and Morell, A. G., Adv. Enzymol. *41* (1974) 99.
4 Beuth, J., Ko, H. L., Oette, K., Pulverer, G., Roszkowski, K., and Uhlenbruck, G., J. Cancer Res. clin. Oncol. *113* (1987) 51.
5 Kieda, C., and Monsigny, M., Invasion Metastasis *6* (1986) 347.
6 Chatterjee, S. K., and Kim, U., J. natl Cancer Inst. *61* (1978) 151.
7 Dennis, J. W., and Kerbel, R. S., Cancer Res. *41* (1981) 98.
8 Vischer, P., and Reutler, W., Eur. J. Biochem. *84* (1978) 363.
9 Lazo, J. S., Hwang, K. M., and Sartorelli, A. C., Cancer Res. *37* (1977) 4250.
10 Wallack, M. K., Brown, A. S., Rosato, E. F., Rubin, S., Johnson, J. L., and Rosato, F. E., J. surg. Oncol. *10* (1978) 39.
11 Shah-Reddy, J., Kessel, D., and Mirchandani, J., Clin. Res. *27* (1979) 585.
12 Kessel, D., and Bauer, C. H., Science *204* (1979) 647.
13 Pulverer, G., Beuth, J., Ko, H. L., Yassin, A., Ohshima, Y., Roszkowski, K., and Uhlenbruck, G., J. Cancer Res. clin. Oncol. *114* (1988) 217.
14 Liotta, L. A., Kleinerman, J., and Saidel, G. M., Cancer Res. *36* (1976) 889.
15 Douglas, W. H. J., and Kaighn, M. E., In Vitro *10* (1974) 230.
16 Hill, R. P., and Bush, R. S., Int. J. Radiat. Biol. *15* (1969) 435.
17 Tsubura, E., Yamashita, T., and Higushi, Y., Cancer Invasion Metastasis (1977) 367.
18 Parish, C. R., Coombe, D. R., Jakobsen, K. B., Bennett, F. A., and Underwood, P. A.. Int. J. Cancer *40* (1987) 511.
19 Coombe, D. R., Parish, C. R., Ramshaw, I. A., and Snowden, J. M., Int. J. Cancer 39 (1987) 82.
20 Fidler, I. J., and Hart, I. R., Science *217* (1982) 998.
21 Dennis, J. W., and Laferte, S., Cancer Metastasis Rev. *5* (1987) 185.
22 Beuth, J., Oette, K., and Uhlenbruck, G., Lectins *5* (1986) 229.
23 Schwartz, R., Schirrmacher, V., and Mühlradt, P. F., Int. J. Cancer *33* (1984) 503.
24 Rauvala, H., and Hakomori, S. I., J. Cell Biol. *88* (1981) 149.
25 Barondes, S., A. Rev. Biochem. *50* (1981) 838.
26 Goldstein, I., Hughes, R. C., Monsigny, M., Osawa, T., and Sharon, N., Nature *285* (1980) 66.
27 Uhlenbruck, G., Beuth, J., Oette, K., Schotten, T., Ko, H. L., Roszkowski, K., Roszkowski, W., Lütticken, R., and Pulverer, G., Dt. med. Wschr. *111* (1986) 991.
28 Beuth, J., Ko, H. L., Schirrmacher, V., Uhlenbruck, G., and Pulverer, G., Clin. exp. Metastasis *6* (1988) 115.

29 Johnson, W. J., Pizzo, S. V., Imber, M. J., and Adams, D. O., Science *218* (1982) 574.
30 Fidler, I. J., Cancer Res. *45* (1985) 4714.
31 Beuth, J., Ko, H. L., Uhlenbruck, G., and Pulverer, G., J. Cancer Res. clin. Oncol. *113* (1987) 359.
32 Gabius, H. J., Engelhardt, R., Cramer, F., Batge, R., and Nagel, G. A., Cancer Res. *45* (1985) 253.

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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

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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,