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# Cell Surface Glycosaminoglycans of a Tumor Cell Line and its DNA Transfected Variant Differing in their Lung Colonizing Potential

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**A highly lung-colonizing cell line RMS/8[2] was obtained by DNA transfection from a low lung-colonizing line RMS/8, a clone of a rat rhabdomyosarcoma cell line. The cells were metabolically labeled with $^{3}$H-glucosamine and $^{35}$S-sulfate. The newly synthesized pericellular glycosaminoglycans and the ability of the cells from the two lines to degrade extracellular matrix components were studied comparatively. The following conclusions were obtained: 1) The *in vitro* proliferation rate is not a determinant in the modulation of the colonizing potential of these cells; 2) The strongly colonizing RMS/8[2] cells release more radioactivity from the radiolabeled extracellular matrix than their weakly colonizing counterparts; 3) The cells with a high colonizing potential incorporated less radioactivity into the cell surface glycosaminoglycans, and exhibited a lower heparan sulfate to chondroitin sulfate ratio than the weakly colonizing RMS/8 line.**

Cell surface properties are often markedly altered as a result of neoplastic transformation [1-5]. We have previously shown that biosynthesis and excretion of the macromolecular glycoconjugates in strongly metastatic and weakly metastatic rhabdomyosarcoma (RMS) cells are different [6]. Weakly metastatic cells as well as non-tumorigenic L6 rat myoblasts were richer in cell surface proteoglycans and glycoproteins than cells from several strongly metastatic rhabdomyosarcoma lines [6, 7].

It has also been reported that highly metastatic cells have an increased ability to digest extracellular matrix (ECM), unlike their counterparts with low metastatic potential [8-12]. The strongly invasive cell lines used in our earlier studies had very different

**Abbreviations:** RMS, rhabdomyosarcoma; ECM, extracellular matrix; GAGs, glycosaminoglycans; DMEM, Dulbecco's modified essential medium; FCS, fetal calf serum; PBS, phosphate buffered saline; CPC, cetylpyridinium chloride.

growth properties (reduced doubling time) when compared with a weakly metastatic RMS/8 cloned cell line. Thus it could not be excluded that the differences in the biosynthesis of cell surface glycosaminoglycans (GAGs) and the increased solubilization of the ECM were at least partially related to the different proliferation rates of these cells.

Recently, we have obtained a high lung-colonizing cell line RMS/8$^2$ by DNA transfection of the low lung-colonizing line RMS/8 previously studied [6, 7]. These two cell lines conserved similar cell morphology and growth kinetics. Therefore it was possible to investigate the biosynthesis of cell surface GAGs and the ability to solubilize the ECM by these two lines, avoiding any interference due to different growth rates.

## Materials and Methods

### *Materials*

Dulbecco's modified essential medium (DMEM), antibiotic-antimycotic mixtures, fetal calf serum (FCS), and geneticin (G-418 sulfate) were obtained from Gibco Ltd. (Paisley, Scotland). The recombinant plasmid pKOneo was a gift of D. Hanahan (CSHL, Cold Spring Harbor, USA). The radioactive precursors $^{35}SO_4$ (535 Ci/mmol) and $^3$H-glucosamine (32.5 Ci/mmol) were purchased from Amersham International (Amersham, UK). Eye-derived growth factor (EDGF) was kindly provided by Y. Courtois (INSERM U 118, Paris, France).

Chondroitin ABC lyase (EC 4.2.2.4) and hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.15) were from Mallet Chem. S.A. (Paris, France). Heparin was from Evans Laboratories (Greenford, Middlesex, UK), pronase from Sigma (St. Louis, MO, USA), DEAE Trisacryl from IBF (Paris, France).

### *Animals*

Female Wistar AG rats (8-10 weeks old) from the breeding animal house of the Institut de Recherches sur le Cancer, Villejuif, France, and maintained in specific pathogen free conditions, were used as recipients for i.v. injection of tumor cells. Standard diets and drinking water were given *ad libitum*.

### *Cell Lines and Culture Conditions*

Parental tumor cell line RMS9-4/0 was originated from the 9-4 rhabdomyosarcoma induced by i.m. injection of nickel powder in one inbred Wistar AG rat as previously described [13]. The standard conditions of growth were DMEM, 1000 U/ml penicillin, 100 mg/ml streptomycin, supplemented with 10% heat inactivated FCS in 5% $CO_2$/90% humidity at 37°C.

The cell line RMS/8 was derived from the parental cell line RMS9-4/0 by a cloning procedure in liquid medium [14]. The cell line RMS/8$^2$ was obtained by cotransfection of the RMS/8 cells with a recombinant neomycin resistant plasmid pKOneo and the genomic DNA from cultured *Drosophila melanogaster* embryonic cells as carrier DNA [15], using the DNA-calcium phosphate precipitate procedure [16]. About 24 h post-transfection the cells were trypsinized and divided 1:5 in a fresh standard medium containing 1 mg/ml geneticin (G418). Re-feeding of the cultures was done every three days, using the

same medium. The plates with about 250 resistant colonies reached confluency after 16 to 20 days. At this time, cells were trypsinized and pooled. One part of the pool was i.v. injected into animals to determine the lung colonizing potential (see below), and the remaining cells were frozen in liquid nitrogen.

*Growth Kinetics Determination*

Cells ($5 \times 10^4$) were seeded in 35 mm Petri dishes and incubated up to five days in standard medium for RMS/8 cells and the same medium plus geneticin for RMS/8$^2$ cells. The duplicate cultures were harvested by trypsinization on day 2, 3, 4 and 5 and the cells counted in a ZM Coulter Counter.

*Determination of the Lung Colonizing Potential*

Cells from subconfluent cultures were detached by trypsinization, washed once, counted and adjusted to a concentration of $10^6$ per ml in FCS free DMEM. For lung colonization assay, 0.1 ml of cell suspension was injected i.v. into the tail vein of syngeneic rats (6-8 rats per group). The animals were systematically killed 70 days later, or before when they presented respiratory difficulties. Tumoral nodules were counted at the surface of the lungs at the autopsy. The statistical significance was calculated by the non-parametric test of Wilcoxon.

*Separation of the Pericellular Macromolecules*

The cells seeded at $10^4$ per 35 mm Petri dish were incubated for three days in the presence of 20 $\mu$Ci/ml of $^3$H-glucosamine (32.5 Ci/mmol) and 20 $\mu$Ci/ml of $^{35}SO_4$ (535 Ci/mmol). At the end of the labeling period the cells reached confluency. The cell monolayers were washed twice with 1 ml of PBS, (0.1 M potassium phosphate, pH 7.3, in 0.15 M NaCl) and 1 ml of heparin solution was added (100 $\mu$g/ml in PBS). After 10 min incubation at 37°C, the heparin solution containing heparin-extracted products was harvested and designated as heparin extract. Cell monolayers were then rinsed twice with 1 ml of PBS, and 1 ml of trypsin solution (0.25% in PBS) was added for another 10 min incubation at 37°C. The trypsin solution containing trypsin-extracted products was removed. The heparin extracts and trypsin extracts were stored at -20°C.

*Glycosaminoglycan Assay*

The heparin and the trypsin extracts were digested with pronase. After the heat destruction of the enzyme (10 min at 100°C), the glycosaminoglycans were assayed by precipitation (aliquots of 50 $\mu$l) on filter paper with 1% (w/v) cetylpyridinium chloride (CPC) [17]. To separate the macromolecular glycoconjugates, the trypsin extracts were chromatographed [18] on a DEAE-Trisacryl (1 × 10 cm) column, equilibrated with 0.05 M Tris-HCl, pH 7.5 buffer in 4 M urea. The elution was started with 10 ml of this buffer, followed by a linear gradient of 0-1.0 M NaCl prepared from 50 ml of the starting buffer and 50 ml of the same buffer containing 1 M NaCl. The flow rate was 0.5 ml/min, fractions of 1.2 ml were collected, and the radioactivity was measured in 0.2 ml aliquots in a Beckman LS 7500 liquid scintillation counter. The dpm values were calculated automatically using a two channel program. The urea was removed from the pooled

**Table 1.** Evaluation of the lung colonizing potential.

| Cells injected | No. of rats/group | Count of lung colonies | | |
|---|---|---|---|---|
| | | individual | median | p (Wilcoxon test) |
| RMS/8 | 6 | 0; 1; 2; 3; 77; 94[a] | 2.5 | |
| RMS/8$^2$ | 8 | 31; 75; 79; 97; 135; 183; 192; 210 | 116 | $< 0.02$ |

[a] Large fluctuations in lung colony counts (0-98) were also observed in our earlier experiments [Cancer Res (1986) 46:3293]. When calculated from several series (in total 33 rats) the median determined for RMS/8 cells was 2, again significantly ($p < 0.01$) different from that of RMS/8$^2$ cells.

proteoglycan-containing fractions (peaks I, II and III from the ion exchange chromatography) by dialysis against water. The proteoglycans were exhaustively digested by pronase [19] and the degradation products were removed by dialysis. Heparan sulfate was characterized by nitrous acid degradation by the procedure of Shively and Conrad [20] modified by Gamse *et al.* [21]. The reaction mixtures were chromatographed on a Bio-Gel P-4 column (1 × 60 cm), eluted with 0.1 M acetic acid. An aliquot of the degradation products was assayed for macromolecular GAGs by CPC precipitation [17]. Chondroitinase ABC digestion was performed as previously described by Saito *et al.* [22]. Hyaluronic acid was digested by *Streptomyces hyalurolyticus* hyaluronidase at pH 6 [23].

*Preparation of Radiolabeled ECM*

The dishes, coated with ECM produced by bovine corneal endothelial cells, were prepared as described [24]. Briefly, the cells were plated at an initial density of $1 \times 10^5$ cells per Petri dish (35 mm) and cultured in standard medium supplemented with 5% dextran T-40 [19]. EDGF was added every day until preconfluence. The cell layer and ECM were metabolically labeled with $^{35}$S-sulfate and with $^3$H-glucosamine (545 Ci/mmol and 32.5 Ci/mmol, respectively). The $^3$H and $^{35}$S labels were added twice to a final concentration of 20 $\mu$Ci/ml and 40 $\mu$Ci/ml, respectively, on day six and eight of a ten day incubation period. The lysis of the cell layer was achieved by 0.5% (by vol) Triton X-100 in PBS. The dishes were then treated with 0.025 M $NH_4OH$ for 2 min followed by four washings in PBS [24].

*ECM Degradation Assay*

The RMS/8 and RMS/8$^2$ cells were suspended in standard medium ($5 \times 10^5$ cells in 2 ml) and deposited on labeled ECM for five days. The medium was harvested every 24 h and replaced by 2 ml of fresh medium. Several dishes coated with radiolabeled ECM were incubated in the absence of cells to measure the spontaneous release of radioactivity. The radiolabeled components released in the medium as a result of the degradation of proteoglycans and glycoproteins were assayed by liquid scintillation counting of aliquots (1 ml).

## Results

### *Evaluation of the Lung Colonizing Potential*

Table 1 shows that non-transfected cells RMS/8 and transfected cells RMS/$8^2$ strongly differ in their lung colonizing potential (median number of lung nodules 2.5 and 116, respectively). Lung colonizing potentials were evaluated as the number of tumoral nodules enumerated at the surface of the lungs, seven to ten weeks after i.v. injection of the tumor cells.

The time course survival of rats injected with transfected cells RMS/$8^2$ was shorter as compared to rats injected with control cells RMS/8 (Fig. 1).

### *Degradation of the ECM*

The results shown in Fig. 2 revealed that the content of radiolabeled components released in the medium is higher for the RMS/$8^2$ cells as compared with RMS/8 cells. The $^{35}SO_4$-label in the ECM and in the material released by the cells was only about 20% of the $^3$H-glucosamine label. Thus only the $^3$H-radioactivity was considered for the quantitative evaluation of the release (Fig. 2). This augmentation is not dependent on the cell density, since the two cell lines cultivated on ECM have similar growth kinetics.

### *Pericellular Content of Glycosaminoglycans*

The glycosaminoglycan labels of the heparin and of the trypsin extracts of both cell lines are shown in Table 2. The material released with heparin (heparin extract) corresponds to the material associated to the cells by electrostatic forces [25]. The trypsin extract contains the bulk of the radioactive cell surface material [6], partially anchored in the membrane [26]. The surface of the weakly metastatic RMS/8 line is richer in glycosaminoglycan label as compared to that of the RMS/$8^2$ cell line (Table 2). These results are independent of growth kinetics which were found to be similar on a plastic surface (results not shown).

### *Separation and Characterization of the Glycosaminoglycans*

The radiolabeled glycoconjugates were separated on the basis of their charge on DEAE-Trisacryl (Fig. 3). The different peaks were tested for the presence of GAGs by CPC precipitation on filter paper [17]. Glycosaminoglycans were detected in three well separated peaks I, II, III, eluted at 0.15, 0.55 and 0.65 M NaCl concentrations, respectively.

Peak I contained hyaluronic acid (70-80% of the content of this peak was digested by hyaluronidase). This material was not further investigated. Peak II contained the heparan sulfate label. This material was resistant to chondroitinase ABC and to *Streptomyces hyalurolyticus* hyaluronidase and it was labeled with sulfate. Bio-Gel P-4 chromatography (not shown) and CPC precipitation assay of the nitrous acid degradation products indicated that more than 90% of the label was degraded to low molecular weight products corresponding essentially to di- and tetrasaccharides. Peak III contained the galactosaminoglycans. About 90% of this peak was degraded by chondroitinase ABC and it was resistant to *Streptomyces hyalurolyticus* hyaluronidase. Some heparan sulfate (5-8%) was also present in the peak III material.

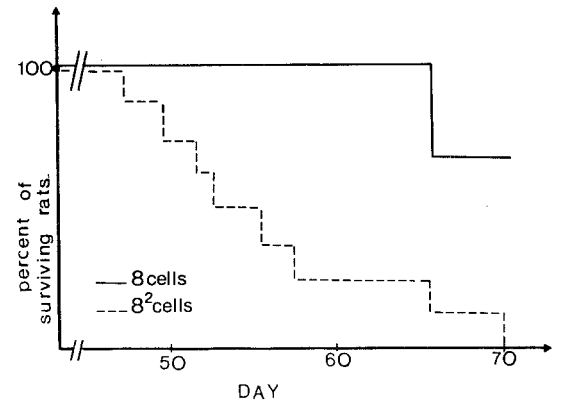


**Figure 1.** Time course of survival of i.v. injected rats. $10^5$ RMS/8 (—) or RMS/$8^2$ (---) cells were injected i.v. into the tail vein of syngeneic rats (6-8 rats per group). The time course of survival of the rats was studied up to 70th day, corresponding to the death of all rats injected with RMS/$8^2$ cells.

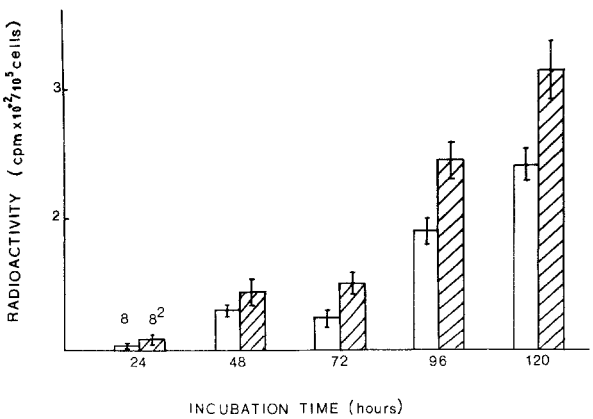


**Figure 2.** $^3$H-Glucosamine label incorporated in the glycosaminoglycans and released by the RMS/8 and RMS/$8^2$ cells from the radiolabeled extracellular matrix deposited by corneal endothelial cells. Background radioactivity counts corresponding to the spontaneous release was substracted from the data obtained. $p < 0.05$ for 24 and 48 h and $p < 0.001$ for the other values. Vertical bars represent the standard deviation, $n = 3$.

The sulfate label of the peak eluted at about 0.25 M NaCl (fractions 30-35, Fig. 3A and 3B) concentration was dialysable and it was not precipitated by CPC. The characterization of this peak was not attempted.

The elution diagrams obtained from the two cell lines RMS/8 and RMS/$8^2$ show that the content of anionic components, eluted with NaCl, is lower for the transfected cell line RMS/$8^2$ (Fig. 3A and 3B). The RMS/$8^2$ cells are particularly poor in galactosaminoglycans (chondroitin and dermatan sulfates) (Fig. 3B).

**Table 2.** Radiolabeled cell surface glycosaminoglycans precipitated by cetylpyridinium chloride from heparin and trypsin extracts of the RMS/8 and RMS/$8^2$ cells. The values listed are averages (+ S.D.) from four different culture dishes.

| | Radioactive label | dpm/$10^6$ cells | |
|---|---|---|---|
| | | RMS/8 | RMS/$8^2$ |
| Heparin extract | $^3H$ | 1353 + 192 | 552 + 48 |
| | $^{35}S$ | 1094 + 210 | 115 + 21 |
| Trypsin extract | $^3H$ | 4730 + 707 | 1380 + 210 |
| | $^{35}S$ | 2424 + 300 | 215 + 18 |

## Discussion

The cell lines RMS/8 and RMS/$8^2$ have different lung colonizing potentials (Table 1) as measured by the number of lung colonies after i.v. injection of tumor cells. Both cell lines are weakly metastatic, i.e. they give rise to rare spontaneous metastases from tumors growing after s.c. injection of cells (results not shown). Furthermore, both lines have identical proliferation rates and their morphological aspects are similar. The identity of the proliferation properties of the RMS/8 and RMS/$8^2$ cells suggests that the *in vitro* proliferative rate is not a determinant in the modulation of the lung-colonizing potential of these cells. The RMS/$8^2$ line was obtained as a pool of neomycin resistant colonies resulting from a cotransfection of the RMS/8 cells with a cloned selectable gene and a genomic eukaryotic DNA. The stable association of the transfected DNA with the host genome is achieved by a random integration into a chromosome of the recipient. Therefore, the resistant colonies, although all expressing the selectable gene, might display a large heterogeneity as to the integration site and a number of integrated copies. The way in which the expression of these genetic modifications might be related to a markedly increased lung-colonizing potential of this population remains unknown.

The main findings of the biochemical investigations on the RMS/8 and RMS/$8^2$ cell lines are as follows. The RMS/$8^2$ cells having a high lung-colonizing potential contained less radioactivity in the cell-associated sulfated GAGs than the low lung-colonizing RMS/8 cells. This result is in agreement with our previous studies, indicating that the label in the cell surface GAGs is higher (trypsin and heparin extracts) in the non-tumorigenic L6 cells or in the weakly colonizing RMS/8 cells, than in the highly colonizing RMS/0, RMS/6 and RMS/J1 lines [6, 7]. The results obtained in this study are compared (Table 3) with earlier data related to the cell surface GAGs of different rhabdomyosarcoma lines. Beside the accumulation of the GAGs on the cell surface, the heparan sulfate to chondroitin sulfate ratio, calculated from the glucosamine labels in the peaks II and III (Fig. 3), is also lower in the strongly colonizing RMS/$8^2$ cells that in the weakly colonizing RMS/8 cells (Table 3). The heparan sulfate-containing fractions (peak II) of both cell types were completely precipitated by CPC even after pronase digestion, and they were almost completely degraded by nitrous acid. Thus the striking differences between the

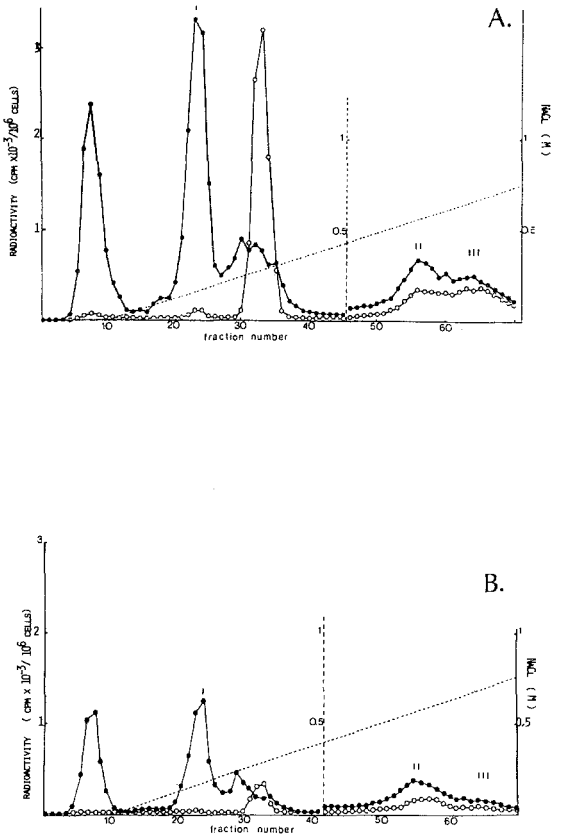


**Figure 3.** Ion exchange chromatography on DEAE-Trisacryl of the cell trypsin extract. A = RMS/8, B = RMS/8$^2$; ● = $^3$H radioactivity; ○ = $^{35}$S radioactivity. Elution was done with a linear gradient of NaCl (0-1 M) in 0.05 M Tris-HCl buffer (pH 7.5) in 4 M urea. (----) = NaCl concentration. The vertical discontinuous line indicates the change in the scale for the cpm values.

$^3$H/$^{35}SO_4$ ratios in the heparan sulfate (peak II, Fig. 3A and 3B) of the two cell lines can be attributed to differences in the heparan sulfate structure and/or metabolism and not to a contamination with glycoproteins.

The tumor cells induce the solubilization and the degradation of macromolecular components of ECM deposited by corneal endothelial cells. This capacity to degrade ECM was correlated with metastatic and colonizing potentials in various types of cells [8-12]. The results presented here are in agreement with these findings, as the strongly colonizing RMS/8$^2$ cells release more radioactivity from the radiolabeled ECM than their weakly colonizing RMS/8 counterparts (Fig. 2). This increase in the release of the label (100%, 60%, and 57% after 72, 96, and 120 h, respectively) is similar to that observed in the case of strongly metastatic RMS [9] or B16 melanoma [12] lines. These data taken together with previous results [8-12] suggest that the involvement of the degradation of the extracellular matrix macromolecules in the tumor spreading is a general phenomenon.

**Table 3.** $^3$H-Glucosamine labels in the cell surface glycosaminoglycans of the non-malignant L6 rat myoblast line and of several rhabdomyosarcoma lines with different colonizing potentials.

| | Cells | | | | | | |
|---|---|---|---|---|---|---|---|
| | L6 | RMS/8 | RMS/8$^2$ | RMS/0 | RMS/13a2 | RMS6 | RMS/J1 |
| Lung colonizing potential[a] | 0<br>0 | 2.5<br>(0-94) | 116<br>(31-210) | 105<br>(34-175) | 132<br>(155-215) | 112<br>(78-190) | 150<br>(105-192) |
| Heparin[b] extract | 7.5 | 1 | 0.35 | 0.58 | 0.32 | 0.41 | 0.49 |
| Trypsin[b] extract | 4.3 | 1 | 0.31 | 0.34 | 0.59 | 0.2 | 0.37 |
| Heparin sulfate/Chondroitin sulfate[c] ratio in the trypsin extracts | 1/0.36[d] | 1/1.3[e] | 1/0.3[e] | 1/0.5[d] | 1/0.8[d] | 1/0.66[d] | 1/0.71[d] |

[a] The median number of lung nodules is given. The numbers in brackets represent limit valves observed in individual animals.

[b] The radioactivity incorporated in the RMS/8 line was taken as unity.

[c] Calculated from the glucosamine labels in the peaks II and III of the DEAE-trisacryl chromatograms, the term chondroitin sulfate includes all the galactosaminoglycans (chondroitin 4- or 6-sulfates and dermatan sulfate).

[d] The values for the lines RMS/0, RMS/6, RMS/13a2 and L6 were calculated from the data published earlier [6, 7]; the data for the RMS/J1 line are unpublished results (F. Redini, E. Moczar, and M.F. Poupon).

[e] The values were calculated from the distribution of the radioactivity in the peaks II and III of the DEAE-trisacryl chromatograms (Fig. 3), (means from two separations from four pooled petri dishes). The differences of the heparan sulfate/chondroitin sulfate ratios obtained in the two separations were less then 6%.

The enzymes degrading the ECM components may also modify macromolecules as collagen or proteoglycans on the surface of the host cells (endothelial or normal blood-borne cells), and facilitate in this way the survival and the arrest of malignant cells in the capillary blood vessels.

It was suggested that the modification of the sugar chains of cell surface glycoconjugates is at least partially dependent on the cell density in culture [27-29]. On the other hand it could not be excluded that the ECM-degrading activity of the different cell lines studied earlier [8, 9] was also modified by differences in the cell densities and by the presence or absence of cell contacts. As the cell densities and the growth kinetics of the RMS/8 and RMS/8$^2$ cells are identical, the effect of these factors on the biosynthesis and/or on the retention of the GAGs on the cell surface and on the degradation of the ECM can be excluded.

The decrease of the label in the cell surface GAGs found in the RMS/8$^2$ cells with the increased colonizing potential confirms our hypothesis on the role of the GAGs in the spread control of the rhabdomyosarcoma cells [6, 7]. Considering these results, it is tempting to speculate that the decrease of the negative charges carried by the GAGs at the surface of the tumor cells could lead to the increase of their aggregating ability. Furthermore, it has been previously demonstrated that highly metastatic and colonizing cells were found to have higher rates of homotypic attachment [30-34].

The variation of the heparan sulfate/chondroitin sulfate ratio indicates that in addition to quantitative differences, the GAGs of the cell lines studied also exhibit qualitative differences related to their colonizing potentials. Thus specific effects, due to defined structural elements of the GAGs, may also be involved in the control of the lung colonization process.


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