Cystic Fibrosis Transmembrane Conductance Regulator Can Export Hyaluronan

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Key Words

ABC transporter · Cystic fibrosis · Cystic fibrosis transmembrane conductance regulator · Extracellular matrix · Hyaluronan

Abstract

Objectives: Hyaluronan, a major water binding component of the extracellular matrix, is synthesised within the cytosol and exported across the plasma membrane by the ABC-transporter MRP5 in fibroblasts. Although its synthesis is vital for embryogenesis, MRP5-deficient mice are without phenotype, suggesting that another transporter had substituted for the MRP5 protein. Thus, we searched for a compensatory exporter in fibroblasts from MRP5 deficient mice and found that cystic fibrosis transmembrane conductance regulator (CFTR) mRNA was upregulated. **Methods:** Hyaluronan export was measured in cell culture. The CFTR transporter was knocked out using si-RNA. Blockers of the ABC-transporter family were used to ascertain the hyaluronan transport capabilities functionally. **Results:** CFTR specific siRNA inhibited hyaluronan export. The tetrasaccharide was exported in undegraded form only from normal human epithelial cells and not from human epithelial cells carrying

ΔF508 CFTR. The CFTR inhibitors GlyH-101 and CFTR₁₇₂ reduced hyaluronan export from CFTR-expressing mouse fibroblasts and from human breast cancer cell lines. Bronchial secretions from patients with cystic fibrosis that consist mainly of necrotic epithelia contained at least 40-fold higher concentration of hyaluronan than secretions from patients with acute bronchitis. **Conclusions:** CFTR transports hyaluronan across the plasma membrane of epithelial cells and this transport mechanism is defective in cystic fibrosis patients.

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Introduction

Cystic fibrosis, the most common severe inherited disorder in Caucasians, is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene which belongs to the ABC transporter family and functions as a chloride channel at apical surfaces of epithelial cells. In the airways, a defective CFTR protein ultimately leads to chronic pulmonary infection and inflammation responsible for almost all the morbidity and mortality of cystic fibrosis patients. Although chloride

transport has been suggested to be the main function of this channel, doubts about this hypothesis arose when Beck et al. [1] found no correlation between chloride currents and cystic fibrosis phenotype. This doubt was reinforced by the observation that secretions from nasal epithelia of cystic fibrosis mice showed decreased volume rather than abnormal salt concentration [2] and that both cystic fibrosis and normal airway surface liquids are nearly isotonic [3]. However, mucous and liquid secretions are uncoupled in cystic fibrosis patients and this uncoupling leads to a defective clearance of mucus from the gland ducts [4]. Thus, CFTR-mediated liquid secretion is normally necessary for the normal hydration of mucins in submucosal glands. In the absence of a sufficient hydration in cystic fibrosis patients, mucus plaques become adherent to airway surfaces [5, 6] and this lack of hydration results in a thick, sticky mucous layer that cannot be removed by cilia and traps bacteria, causing chronic infections.

Hyaluronan is a very large glycosaminoglycan that is exported into the extracellular matrix by both fibroblasts and epithelial cells, where it attracts water, swells accordingly to enormous volumes and displaces other resident macromolecules. Hyaluronan biosynthesis proceeds by alternate transfer of the precursor nucleotide sugars UDP-GlcA and UDP-GlcNAc at the inner face of the plasma membrane [7–10]. The growing hyaluronan chain is synthesised within the cytoplasm and exported into the extracellular matrix. The export was originally thought to be performed by the synthase itself in *Streptococci* [11] as well as in vertebrate cells [12]. However, we discovered that hyaluronan is exported through the protoblast membrane of *Streptococci* by an ABC transporter [13]. As the streptococcal hyaluronan transporter had structural and functional homology to human multidrug resistance transporters, we investigated potential hyaluronan exporters in human fibroblasts and identified MRP5 as its main transporter across the plasma membrane [14, 15]. Our findings thus showed that two cellular processes are essential for the deposition of hyaluronan in the extracellular matrix: hyaluronan synthesis via the hyaluronan synthase within the cytosol, and hyaluronan export through the plasma membrane via the MRP5 transporter.

Lack of hyaluronan deposition in the extracellular matrix is incompatible with life, as demonstrated by hyaluronan synthase-deficient mice, which die at a stage E9.5 during embryonic development [16]. However, if MRP5 was the sole hyaluronan transporter, it would be expected that MRP5-deficient mice die at about the same intra-

uterine age as the hyaluronan synthase deficient mice. As MRP5-deficient mice do not have an obvious phenotype [17] it is likely that they have an alternative hyaluronan exporter from another member of the ABC transporter family. Members of this large family can compensate each other in the pump function [18, 19]. We therefore searched for members of the ABC transporter family which could substitute for the MRP5 transporter in fibroblasts of MRP5 deficient mice. We focused on CFTR as this transporter has previously been shown to transport glucuronic acid [20] which is a moiety of hyaluronan.

Materials and Methods

Sputum was obtained from patients with cystic fibrosis and patients with acute tracheobronchitis. MRP5-deficient mice were provided by Dr. P. Borst, Amsterdam [17]. CFTR and non-sense siRNA were from Ambion (Cambridgeshire, UK); monoclonal antibodies against CFTR were from Acris (Herford, Germany); GlyH-101 (N-(2-Naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)-methylene)-glycine hydrazide) was from Calbiochem; primers were from MWG Biotech (Ebersberg, Germany); the thiazolidone CFTRact-06 was synthesised as described [21]; other chemicals were from Sigma Chemical.

Cells

Primary skin fibroblast cultures from wild-type and MRP5-deficient mice were established and grown in RPMI supplemented with streptomycin/penicillin (100 units of each/ml) and 10% foetal calf serum. Human epithelial cells containing wild-type CFTR and mutant, Δ F508 CFTR were kindly provided by Dr. D.C. Gruenert [22].

General Methods

Determination of hyaluronan synthase activity [14], and hyaluronan concentration by an ELISA-like assay using the hyaluronan binding protein of aggrecan [14] and knockdown by RNA interference [15] were performed as described. For quantitative RT-PCR we used RT-PCR of CFTR, MRP1–6, MRP8, MDR1 and α -actin using the primers in table 1 and the Access RT-PCR System (Promega) and Platinum[®] SYBR[®] Green qPCR SuperMix-UDG (Invitrogen) and a Mastercycler[®] ep realplex (Eppendorf).

Preparation of Fluorescent Hyaluronan and Measurement of Its Export

Hyaluronan (10 mg) was degraded into oligosaccharides by testicular hyaluronidase (1 mg/ml) in 10 ml of 0.1 M sodium acetate buffer pH 5.2, 0.15 M NaCl for 16 h at 37°C, yielding the hyaluronan tetrasaccharide as the main digestion product [23]. The enzyme was inactivated at 100°C for 3 min and precipitated by centrifugation for 3 min at 14,000 rpm. The supernatant was evaporated, and the hyaluronan oligosaccharides were dansylated at the reducing end according to the procedure of Du and Eddington [24]. The residue was dissolved in 100 μ l of 1% trichloroacetic acid, 26.5 mg of dansylhydrazine in 200 μ l of dimethylsulfoxide and the mixture was kept at 60°C for 18 min. The fluorescent hy-

Table 1. Primer and siRNA sequences used**a** The primer sequences of CFTR, MRP 1–6, MRP8 and MDR1 for RT-PCR and expected sizes of PCR products

Gene	Forward primer sequences (5'–3')	Reverse primer sequences (5'–3')	Product bp
β-Actin	CGT GCG TGA CAT CAA AGA GA	TCC TGT CAG CAA TGC CTG GG	341
MDR1	GCA ATT TAG TGG AGT CGT GTT C	GCA AGA CTT AGT GGT GCT AG	640
MRP1	CTG ATG AAG AAG TCT GGA TGG	GTG AGG GCT TGA AGA GGA AA	329
MRP2	TTG GCT CAT CTC AAA TCC TT	ACA ATC TTG CCG CTC TCT AG	304
MRP3	CTA TGC CTT ACA GGT GAC CA	GGG ACA ACA TCA GGT ACT AAT	309
MRP4	ATG TGG ACC CAA GAA CGG AT	GGC TGT CCA TTG GAG GTG TT	327
MRP5	CAT AGC AAG AGC AAG AGC CC	GTG GTA ATC AGG GCA ATG CT	402
MRP6	GGA TGA ACC TGG ACC TGC TT	TGC CCC TCA TCC ATG ACT AGG	463
CFTR	AGT GAC CTT ACA AGA ATG GAG G	GGC AAT CCA ACA TCG CTT CTA	403
MRP8	CGT TCA CGC CTC TCC ATC	AGT TGC TTC GTC CAT GAT GCT	281

b CFTR sense and antisense sequences (5'–3') for siRNA; sequences 4 and 5 are mismatch (underlined) siRNA of sequences 2 and 3, respectively, that were used as controls

No.	Sense	Antisense
1	GCAGAGUGUUUAAGUUUAUtt	AUAAACUAAAACACUCUGCtc
2	GCUGAAAAUGACCCGGAAGtt	CUUCCGGGUGAUUUUCAGCtc
3	GGCGGCCUUAUAGAGGUUCtt	GAACCUCAUAUAGGCCGCCtt
4	GCUGAAA <u>GUG</u> CCCGGAAGtt	CUUCCGGG <u>CC</u> CA <u>CU</u> UUCAGCtc
5	GGCGGCCU <u>GUG</u> AGAGGUUCtt	GAACCUCA <u>CA</u> <u>CA</u> AGGCCGCtt

aluronan oligosaccharides were purified by gel filtration on a Sephadex G-15 column (2.4 × 12 cm) using the pinocytosis medium (Dulbecco's medium containing 1 M sucrose, 10% polyethylene glycol 600) as eluent. The eluate was monitored by a fluorescent lamp. The first fractions of the void volume were discarded and the highly fluorescent fractions were collected.

Wild-type and mutant ΔF508 CFTR human epithelial cells (2 × 10⁶) were seeded in 6-well plates (9.6 cm² surface area per well) and grown for 24 h. The medium was withdrawn and the cells were incubated in 1 ml/well of pinocytosis medium containing the fluorescent hyaluronan oligosaccharides for 15 min at 37°C [25]. This procedure has been shown to maintain excellent cell viability [26, 27], plating efficiency and minimal damage to the cells [28]. Non-specific effects of the method were confined to transient decrease of protein and DNA synthesis [29]. Further possible non-specific effects have already been excluded in our previous publication [15]. The pinocytosis medium was then replaced with a mixture of Dulbecco's medium/H₂O 1:2 (v/v) for 5 min to lyse the pinocytotic vesicles. This loading procedure was repeated twice. Cells were incubated in PBS containing 5.6 mM glucose, 1 mM MgCl₂ and 5 mM KCl. After different time periods, the appearance of fluorescence in the supernatant was determined in a fluorimeter with the excitation wavelength at 330 nm and emission wavelength at 530 nm. The sensitivity of the fluorimeter was calibrated by dansylcadaverine. The fluorescent solution (2 ml) was then lyophilised, the residue was dissolved in 0.1 ml of water and applied to gel filtration on Sephadex-G15 using a Pasteur pipette.

Fractions of 0.2 ml were collected and the fluorescence was determined in a fluorimeter. As a specificity control, we used 1 mg of glucose tetrasaccharide dansylated by the same technique as described above and subjected it to the same export procedure.

Determination of Cell-Associated Hyaluronan

Fibroblasts were seeded into 24 well plates (1 cm² surface area per well) and grown to confluency. The medium was replaced with 1 ml of medium containing increasing concentrations of GlyH-101 and [³H]glucosamine (1 μCi/ml) and incubated for 24 h at 37°C. The supernatant was discarded, the cells were washed with PBS, lysed directly with 0.1% SDS or incubated with PBS containing 100 units/ml of hyaluronidase for 1 h and then lysed with 0.1% of SDS. The lysate was applied to descending paper chromatography in a mixture of 1 M ammonium acetate, pH 5.5, and ethanol (7:13) for 24 h. The origin was excised and the radioactivity was determined. Incorporation of radioactivity into [³H]hyaluronan was calculated from the difference in the absence and presence of hyaluronidase.

Determination of Hyaluronan Concentration in Bronchial Secretions

The secretions were weighed and dissolved in a 10-fold amount of 4 M guanidinium hydrochloride. Undissolved material was removed by centrifugation at 14,000 g for 3 min. Aliquots of the supernatant were directly used for determination of the hyaluronan concentration as described [14].

Fig. 1. Expression of multidrug-resistant transporters in wild-type and MRP5-deficient mouse fibroblasts. **a** Qualitative screening of MDR1, MRP1–6, CFTR and MRP8 mRNA of wild-type (wt) and MRP5-deficient mouse fibroblasts (ko). Cellular mRNAs were isolated and the presence of mRNA was determined by RT-PCR. **b** Levels of CFTR expression. mRNAs were isolated from wild-type and MRP5-deficient mouse fibroblasts and quantitative real-time PCR was performed with CFTR-specific primers, actin primers as control. Three different samples were analysed for each assay.

Results

Identification of the Hyaluronan Exporter in MRP5-Deficient Mouse Fibroblasts

For the identification of the hyaluronan exporter in MRP5-deficient mouse fibroblasts, we isolated and cultured primary fibroblasts from MRP5-knockout mice [17]. To determine whether the loss of MRP5 caused changes in the expression pattern of other members of the ABC transporters, RT-PCR analyses of MRPs 1–6, CFTR, MRP8 and MDR1 were performed with wild-type and MRP5-deficient fibroblasts. mRNAs were amplified individually using primers specific for each transporter, and the levels of corresponding PCR products were visualised on an agarose gel (fig. 1a). The identity for the PCR products for all the transporters was verified by their molecular size and by nucleotide sequencing. The results showed that the levels of MDR1, MRP1, MRP2, MRP3, MRP6 and MRP8 appeared to be very low or non-detectable. MRP5 appeared unchanged, but no MRP5 protein was detected in Western blots with antibodies against MRP5 confirming previous results [17]. The greatest dif-

ference between wild-type and knockout fibroblast was noticed for CFTR. It was virtually absent in wild-type fibroblasts and prominent in MRP5 knockout fibroblasts. A Western blot showed that CFTR was detectable in MRP5-deficient cells, but not in wild-type cells (data not shown).

Inhibition of Hyaluronan Export from MRP5-Deficient Mouse Fibroblasts by CFTR-siRNA

To determine the mRNA levels of CFTR in wild-type and MRP5-deficient fibroblasts, we used quantitative RT-PCR with CFTR-specific primers (table 1). Figure 1b shows that the level of CFTR expression was much higher in MRP5-deficient fibroblasts than in wild-type mouse fibroblasts.

To assess the role of CFTR in hyaluronan export, MRP5-deficient mouse fibroblasts were transfected with three CFTR siRNA in different combinations and non-sense siRNA as control (table 1). Initial experiments indicated that 20 nM of CFTR-specific siRNA was the optimal concentration for reduction of CFTR mRNA. Western blot analysis of whole cell lysates with monoclo-

nal antibodies against CFTR showed knockdown of the 170-kDa protein with siRNA to different extents (fig. 2a). When the three siRNAs were used individually, only siRNA 3 was effective and this effect was enhanced by combination with siRNA 1 or 2. In contrast, the combination of siRNA 1 and 2 had no effect. Therefore, the effect of CFTR knockdown was measured with the mixtures of siRNA 2 + 3 and 1 + 3. Figure 2b shows that hyaluronan export was slightly reduced by siRNA3 and significantly reduced by the combined application of siRNA 1 + 3 or 2 + 3, as compared with non-sense siRNA. In control experiments, we analysed CFTR siRNA on wild-type cells as well as unrelated siRNA in MRP5-deficient cells and did not find any effects (data not shown). As an additional control, we performed control experiments with mismatch siRNAs 4 and 5 which showed that hyaluronan secretion was not affected. As a further control, we analysed the stability of hyaluronan in the culture supernatant and found that it was not degraded (data not shown), indicating that the effects measured depended solely on hyaluronan acid secretion and not degradation. These siRNAs did not reduce the CFTR message, the protein expression and hyaluronan export (data not shown). This indicated that CFTR exported hyaluronan in MRP5-deficient mouse fibroblasts over-expressing CFTR. To analyse, whether the replacement of MRP5 by CFTR has any effect on the expression or activity of the hyaluronan synthase, we isolated membranes from wild-type and knockout fibroblasts and found that the specific activity of the synthase was not altered. The membrane fractions isolated from 5×10^7 wild-type or MRP5-deficient fibroblasts incorporated $39,945 \text{ cpm} \pm 3,478 \text{ cpm}$ or $39,691 \text{ cpm} \pm 844 \text{ cpm}$ from UDP- ^{14}C GlcA within 3 h at 37°C into ^{14}C hyaluronan, respectively.

Effect of CFTR Inhibition and Activation on Hyaluronan Export

If hyaluronan was exported from MRP5-deficient fibroblasts by CFTR, the inhibitory profile should change between normal fibroblasts and MRP5-deficient fibroblasts. Therefore we compared the inhibitory profiles of GlyH-101, a CFTR-specific inhibitor of chloride export [30], on hyaluronan export from wild-type and MRP5-deficient mouse fibroblasts. Figure 3a shows that GlyH-101 inhibited hyaluronan export from MRP5-deficient fibroblasts at concentrations between 1.5 and $25 \mu\text{M}$ more effectively than from wild-type fibroblasts. It appeared that GlyH-101 could specifically differentiate between the export of hyaluronan by MRP5 and by CFTR. At con-

Fig. 2. Inhibition of hyaluronan export by CFTR-siRNA in MRP5-deficient mouse fibroblasts. Fibroblasts were seeded in a 12-well plate and incubated overnight. Cells at 50–60% confluence were transfected with a mixture of the siRNAs, as compared with a non-sense siRNA. **a** Specific inhibition of protein expression. The relative intensities of the CFTR protein at 170 kDa were determined by Western blotting with a CFTR-specific monoclonal antibody. **b** Inhibition of hyaluronan export. The supernatants were withdrawn after 48 h (open bars) and 72 h (solid bars) for determination of hyaluronan concentration. The error bars indicate the SD of 3 experiments. For comparison, the amount of hyaluronan produced by wild-type cells was $9 \mu\text{g/ml}$ after 48 h, and $13 \mu\text{g/ml}$ after 72 h. The mixtures of CFTR siRNA 1 + 3 or 2 + 3 significantly reduced hyaluronan export. * $p < 0.02$, ** $p < 0.002$ (ANOVA test).

centrations above $50 \mu\text{M}$, it abolished hyaluronan export from both cell lines and it is possible that it exerted non-specific side effects. As a verification of this hypothesis we compared the accumulation of intracellular hyaluronan in the cell lines. Figure 3b shows that intracellular hyaluronan accumulated in MRP5-deficient fibroblasts with increasing concentrations of GlyH-101, but not in wild-type fibroblasts. At a low-medium concentration of GlyH-101, where extracellular hyaluronan acid was reduced, there was no corresponding significant increase observed in the intracellular fraction. In a control exper-

Fig. 3. Inhibition and activation of hyaluronan export. Equal numbers of wild-type (□) and MRP5-deficient (■) mouse fibroblasts were cultured with increasing concentrations of GlyH-101, a specific inhibitor of chloride export by CFTR, for 24 h. The media were replaced with fresh media containing identical GlyH-101 concentrations, and after an additional 24 h of incubation the amount of extracellular (a) and intracellular (b) hyaluronan was determined and plotted as the SD of 3 measurements. The 100% values for [^3H]hyaluronan for 10^6 wild-type cells corresponded to 2.3×10^6 dpm for the extracellular and 3.6×10^5 dpm for the intracellular compartment, and for 10^6 MRP5-deficient cells to

1.9×10^6 dpm for the extracellular and 4.2×10^4 dpm for the intracellular compartment. Hyaluronan export was also tested with increasing concentrations of CFTR_{act}-06 (c), an activator of chloride export, and the amount of extracellular hyaluronan was determined as in figure 2b and plotted as the SD of 4 measurements. Hyaluronan export was also inhibited from the breast cancer carcinoma cell line HMT3552 by increasing concentrations of GlyH-101 (□) or CFTR₁₇₂ (■) (d). The results were normalised to the control without inhibitors containing 2 $\mu\text{g/ml}$ of hyaluronan in the culture medium. The error bars indicate the SD of 3 determinations.

iment, we found that GlyH-101 had no effect on the hyaluronan synthase activity as measured on an isolated membrane preparation (data not shown).

In addition, we also analysed the effect of CFTR_{act}-06, a specific CFTR activator that greatly prolongs the channel open state [31, 32], on hyaluronan export. Figure 3c shows that CFTR_{act}-06 stimulated hyaluronan export only from MRP5-deficient fibroblasts, but not in wild-type fibroblasts.

It would be desirable to analyse the effects of the CFTR inhibitors on hyaluronan export from natural epithelial cells directly. Unfortunately, the cell lines did not express hyaluronan synthase. Therefore we used breast cancer carcinoma cell lines that express CFTR [33] and produce hyaluronan [34]. Figure 3d shows that both CFTR inhibitors GlyH-101 and CFTR₁₇₂ reduced hyaluronan export from HMT3552 cells in a concentration-dependent manner. Similar inhibitions were found for other cell lines.

Fig. 4. Export of fluorescent hyaluronan oligosaccharides. Normal human epithelial cells ($\square$) and human epithelial cells carrying Δ F508 CFTR ($\blacksquare$) were loaded with dansylated hyaluronan oligosaccharides by osmotic lysis of pinocytotic vesicles and the export was measured by fluorescence in the culture supernatant at the incubation periods indicated. As further controls, normal human epithelial cells were loaded with dansylated glucose tetrasaccharide containing α 1–4 linkages ($\triangle$), with dansylcadaverin (∇), or with dansylated hyaluronan oligosaccharides in the presence of the CFTR inhibitor GlyH-101 ($\star$). The error bars indicate the SD of 3 experiments. * $p < 0.2$, ** $p < 0.01$ (ANOVA test).

Export of Fluorescent Hyaluronan Oligosaccharides from Human Epithelial Cells

It would be desirable to measure the export of hyaluronan by CFTR in reconstituted vesicles in order to eliminate any possible intracellular metabolic interference. However, this approach was unsuccessful, probably due to the lack of intracellular hyaluronan-binding proteins. We therefore compared the export of fluorescent hyaluronan oligosaccharides introduced into the cytosol by osmotic lysis of pinocytotic vesicles from normal human epithelial cells and human epithelial cells carrying mutated Δ F508 CFTR. These cell lines differ only in the presence or absence of F508, rendering CFTR functionally inactive. These cells did not express MRP5 in a Western blot (data not shown). The appearance of fluorescence in the culture supernatant was measured after different incubation periods. Figure 4 shows that only native human epithelial cells with intact CFTR, but not the functionally inactive Δ F508 CFTR cells, exported hyaluronan oligosaccharides. The exported dansylated product was analysed by gel filtration on Sephadex-G15 and showed an identical elution position as the original dansylated hyaluronan tetrasaccharide, indicating that no intracellular degradation had occurred. As a control for the ef-

Table 2. Analysis of hyaluronan from bronchial secretions of patients with cystic fibrosis and patients with bronchitis

	μg hyaluronan/ g secretion	mg protein/ g secretion	μg hyaluronan/ mg protein
<i>Acute bronchitis patients</i>			
1	0.20 ± 0.04	0.29 ± 0.04	0.70
2	0.05 ± 0.02	0.16 ± 0.03	0.31
3	0.07 ± 0.02	0.32 ± 0.02	0.22
4	0.03 ± 0.01	0.08 ± 0.01	0.37
5	0.02 ± 0.01	0.15 ± 0.02	0.13
6	0.03 ± 0.01	0.14 ± 0.01	0.22
7	0.32 ± 0.02	0.38 ± 0.02	0.84
<i>Cystic fibrosis patients</i>			
CF 772	188 ± 9.5	2.44 ± 0.11	77
CF 785	784 ± 10.9	3.95 ± 0.01	198
CF 789	638 ± 54.1	5.31 ± 0.98	120
CF 791	710 ± 35.8	8.76 ± 0.47	81
CF 792	384 ± 26.1	2.31 ± 0.23	166
CF 798	124 ± 16.9	3.54 ± 0.17	35

Data are means $\pm$ SD of 3 measurements.

fectiveness of the procedure, we introduced dansylcadaverin into the cytosol. Its fluorescence could easily be detected in the cells, but it did not appear in the culture supernatant. As a further specificity control, we performed the experiment with dansylated glucose tetrasaccharide and could not detect any fluorescence in the culture supernatant indicating that export was hyaluronan specific.

Hyaluronan Content in Bronchial Secretions

The hyaluronan concentrations were determined in bronchial secretions from 6 patients with cystic fibrosis and 7 patients with bronchitis. It could only be detected in the thick mucus from cystic fibrosis patients after dissociation with 4 M guanidinium hydrochloride. The data were related to weight as well as to protein concentrations. Table 2 shows that the hyaluronan concentrations in cystic fibrosis patients ranged from 124–788 $\mu\text{g/g}$ of secretions or 35–198 $\mu\text{g/mg}$ of protein as compared to secretions of bronchitis patients with 0.02–0.32 $\mu\text{g/g}$ of secretions or 0.13–0.84 $\mu\text{g/mg}$ of protein. Thus the ratios of hyaluronan to protein concentrations were at least 40-fold higher in all cystic fibrosis patients.

Discussion

Transmembrane transporters of the ATP-binding cassette gene family are the largest family of transport proteins. As these transporters have evolved from a common ancestor gene, a considerable degree of molecular redundancy concerning the transport capabilities are to be expected. Here we were able to demonstrate that the primary hyaluronan exporter MRP5 is readily replaced by a secondary hyaluronan exporter, namely CFTR, in MRP5-deficient mice.

The hyaluronan transport function of CFTR was ascertained using different methods and experimental approaches. First, quantitative RT-PCR revealed that the expression of the CFTR mRNA was up-regulated in fibroblasts from MRP5-deficient mice (fig. 1a). To show that CFTR expression was indeed related to hyaluronan export, we used CFTR-specific siRNA. Fibroblasts derived from MRP5-deficient mice transfected with CFTR-specific siRNA showed a decrease in hyaluronan deposition in the extracellular matrix as compared to the non-sense siRNA control (fig. 2b). As functional MRP5 is not available as a hyaluronan transporter in MRP5-knockout mice, our results indicated that the upregulated CFTR compensates MRP5 for hyaluronan export in MRP5-deficient mice. The inhibition of hyaluronan export by GlyH-101 and activation by CFTR_{act}-06, an inhibitor and activator of CFTR, respectively, for chloride transport, further supports our hypothesis (fig. 3). Since normal epithelial cell lines did not produce any hyaluronan in cell culture, we wanted to expand our hypothesis that CFTR exports hyaluronan on epithelial cells that produce hyaluronan on their own. We analysed breast cancer cell lines that originate from epithelia, express CFTR [33] and produce hyaluronan [35] and found that both CFTR inhibitors GlyH-101 and CFTR₁₇₂ reduced hyaluronan export. Our result that CFTR exports hyaluronan corroborates recent data that failed to detect MRP4 or MRP5 as principle hyaluronan exporters in breast cancer cells [34]. This result is of interest for two reasons. First, it indicates that a tissue-specific hyaluronan transport exists: mesenchymal cells including fibroblasts use MRP5 as the principle hyaluronan transporter, while epithelia use CFTR as the principle hyaluronan transporter. Second, it is interesting to note that a defect in CFTR inhibits breast cancer cell growth [36] and that silencing to the hyaluronan synthase suppresses the invasiveness of breast cancer cells [37]. Our finding would therefore combine these hitherto unlinked observations as both hyaluronan synthesis catalysed by hyaluronan synthase and its export by ABC-

transporter are necessary to result in a deposition of hyaluronan in the extracellular matrix. Impairment of both mechanisms results in reduced growth of breast cancer.

GlyH-101 is known to clog CFTR from outside and thus cannot influence intracellular metabolism [30]. The observation that GlyH-101 inhibited the accumulation of intracellular hyaluronan more than of extracellular hyaluronan could be explained by a feedback inhibition of the hyaluronan chains on their own elongation that we described previously [38]. The inhibitory constant K_i for chloride export was 1.4 μ M [30], and thus is in the same concentration range as for the inhibition of hyaluronan export (fig. 3a). In addition, fluorescent hyaluronan oligosaccharides were only exported from human epithelial cells with intact CFTR, but not from mutated Δ F508 CFTR cells.

At first glance, it is surprising that such diverse substrates as chloride and hyaluronan are exported by the same protein, even though ABC transporters have notoriously promiscuous properties. However, since CFTR also exports glucuronic acid, one of the two monosaccharide components of hyaluronan disaccharide units [39], comparison of protein sequences of the ABC transporter family revealed that CFTR is one of the closest relatives to MRP5 [14], further underpinning the common properties of the two proteins. Thus, we conclude that CFTR is not only responsible for chloride, but more importantly for hyaluronan export. This fact has important consequences for the understanding of CFTR in the pathophysiology of cystic fibrosis.

Hyaluronan plays a major role in mucosal defence because it increases the ciliary beat frequency of tracheal epithelial cells [40, 41]. It is a constituent of human airway secretions and is produced mostly by ciliated surface epithelial cells, with some contributed by submucosal glands [42–44]. In these inflammatory diseases, hyaluronan is exported from serous, but not mucous cells, of human nasal and tracheobronchial submucosal glands only after parasympathetic stimulation [45]. This observation corroborates the finding that CFTR is primarily expressed on the apical surface of serous cells in bronchial submucosal glands and on ciliated cell on the epithelial surface [46–48]. Interestingly, it has also been observed that CFTR gene transfer corrects for defective secretion of unspecified glycoconjugates from tracheal epithelial cells of patients with cystic fibrosis [49].

Hitherto, the hyaluronan concentrations in sputum have only been analysed from healthy subjects, asthma and rhinitis patients [50], and chronic obstructive pulmonary airway disease patients [51]. The analysis of lung secretions from patients with cystic fibrosis revealed that

hyaluronan was the only glycosaminoglycan found in detectable amounts [52]. We analysed for the first time the hyaluronan concentrations in bronchial secretions from patients with cystic fibrosis and compared it with secretions from patients with acute bronchitis. Bronchitis sputum contained similar amounts of hyaluronan as other inflammatory tracheal diseases. However, the hyaluronan concentrations in cystic fibrosis sputum ranged from 124 to 784 µg/g and were thus more than 100-fold higher than in acute bronchitis. The high hyaluronan concentrations were also reflected in the hyaluronan to protein ratio. Hyaluronan could only be detected after solubilisation under dissociative conditions with 4 M guanidinium hydrochloride. At first sight, our results that defective hyaluronan export leads to higher hyaluronan concentrations in cystic fibrosis sputum seem counter-intuitive as one would expect lower levels. However, the cystic fibrosis sputum mainly consists of cellular debris from necrotic epithelia. Major components of cystic fibrosis sputum are DNA and actin filaments originating from necrosis of epithelial cells [53, 54] that have an increased cell death rate [55] and a more than 85-fold higher proliferation rate

than normal epithelium [56]. The intracellular accumulation of hyaluronan in F508 epithelial cells may lead to swelling and oncosis that is caused by defective anion channels [57]. We assume that hyaluronan in sputum from cystic fibrosis patients is associated with intracellular proteins which prevent full hydration. This lack of hydration results in the typical thick mucous seen in cystic fibrosis patients. Our results may be a compromise in the controversy of the cystic fibrosis pathology between the hydration and the salt hypothesis [58], because hyaluronan fulfils both properties being an extremely hydrated salt. Aggregation of hyaluronan with binding proteins has been suggested to be a reaction that accompanies inflammation [59].

Acknowledgments

The authors thank Dr. S. Misra and Prof. Dr. J.E. Scott for critical reading of the manuscript, and A. Blanke, R. Gehrke, U. Rasmussen, K. Siebert, and R. Schulz for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 492).

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