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Adsorption of Glycosaminoglycans to the Cell Surface Is Responsible for Cellular Donnan Effects

Daniel Hagenfeld,[1] Nadine Kathagen,[2] and Peter Prehm[2,3]*

[1]*Section of Periodontology, Department of Conservative Dentistry, Clinic for Oral, Dental and Maxillofacial Diseases, University Hospital Heidelberg, Heidelberg, Germany*

[2]*Muenster University Hospital, Institute of Physiological Chemistry and Pathobiochemistry, Waldeyerstr. 15, D-48129 Muenster, Germany*

[3]*Hylitis, Rudolf-Harbig-Str. 5, D-48301 Nottuln, Germany*

ABSTRACT

In previous publications, we showed that extracellular glycosaminoglycans reduced the membrane potential, caused cell blebbing and swelling and decreased the intracellular pH independently of cell surface receptors. These phenomena were explained by Donnan effects. The effects were so large that they could not be attributed to glycosaminoglycans in solution. Therefore, we tested the hypothesis that glycosaminoglycans were concentrated on the cell membrane and analysed the mechanism of adsorption by fluorescent hyaluronan, chondroitin sulphate and heparin. The influence of the CD44 receptor was evaluated by comparing CD44 expressing human fibroblasts with CD44 deficient HEK cells. Higher amounts of glycosaminoglycans adsorbed to fibroblasts than to HEK cells. When the membrane potential was annihilated by substituting NaCl by KCl in the medium, adsorption was reduced and intracellular pH decrease was abolished. To eliminate other cellular interfering factors, potential-dependent adsorption was demonstrated for hyaluronan which adsorbed to inert gold foils in physiological salt concentrations at pH 7.2 and surface potentials up to 120 mV. From these results, we conclude that large cellular Donnan effects of glycosaminoglycans results from receptor mediated, hydrophobic and ionic adsorption to cell surfaces. J. Cell. Biochem. 115: 1334–1341, 2014.

KEY WORDS: HYALURONAN; PROTEOGLYCANS; DONNAN EFFECT; EXTRACELLULAR MATRIX

The glycosaminoglycans hyaluronan, chondroitin sulphate and heparin are generally considered to interact with cells by binding to the cell surfaces. CD44 is the principle hyaluronan receptor [Aruffo et al., 1990; Lüke and Prehm, 1999], but has also affinity for chondroitin sulphate [Kawashima et al., 2000] and heparin [Jalkanen and Jalkanen, 1992].

There are only few reports that glycosaminoglycans also bind by hydrophobic forces despite their pronounced hydrophilicity. Hyaluronan has hydrophobic areas for self-aggregation [Scott et al., 2003] and adsorbs to hydrophobic resins [McCourt and Gustafson, 1997] and phospholipids [Wang et al., 2013]. Chondroitin sulphate adsorbs to a hydrophobic dye [Hüglin et al., 1986]. Both chondroitin sulphate and heparan sulphate bind to cells by hydrophobic and ionic interactions [David and van den Berghe, 1989; David et al., 1989; Edwards and Wagner, 1992].

Although mammalian cells contain a large hydrophilic glycocalix on the cell surface, they nevertheless adsorb to hydrophobic substrates [Halperin et al., 1984; Bauer et al., 2008]. Cells form a glycocalix and have a net negative charge due to fixed sialic acid residues [Thethi and Duszyk, 1997; Nassimizadeh et al., 2010] and adsorbed of glycosaminoglycans [Maroudas et al., 1969; Van Damme et al., 1994]. But they have a cationic surface layer formed by the membrane potential which is the driving force for hyaluronan export [Hagenfeld et al., 2012] and adsorption of anionic nanoparticles [Shin et al., 2013].

Recently, we demonstrated that extracellular glycosaminoglycans have profound effects on fundamental cellular properties. They reduce the membrane potential [Hagenfeld et al., 2010], alter the cell volume [Joerges et al., 2012] and decrease the intracellular pH [Kathagen and Prehm, 2013]. These phenomena were independent of the CD44 receptor and can be explained by Donnan effects. A Donnan effect describes the unequal distribution of small diffusible ions across a membrane, when one compartment contains a charged non-diffusible macromolecule. The macromolecule causes expulsion

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*Correspondence to: Peter Prehm, Hylitis, Rudolf-Harbig-Str. 5, D-48301 Nottuln, Germany.
E-mail: prehm@uni-muenster.de
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of small ions into the neighbouring compartment. The magnitude of expulsion is determined by the Donnan coefficient which is directly proportional to the charges of the macromolecule and inverse proportional to the concentrations of other small ions in the solution.

The Donnan effects in our experiments were so large that they exceeded by far the theoretical expectations calculated from the concentrations of the glycosaminoglycans in free solution.

In a previous publication, we showed that the glycosaminoglycans hyaluronan, chondroitin sulphate and heparin caused an intracellular $\triangle pH = 0.4$ decrease at concentrations of 0.25 mg/ml [Kathagen and Prehm, 2013]. This converts into a 2.5-fold intracellular increase of H^+ concentration. If the Donnan effect exerted from glycosaminoglycans in solution was the only mechanism, the effect should be related to the concentration of extracellular membrane impermeable ions and can be described by the Donnan coefficient:

$$r_D = 1 + \frac{n[P^n]}{[anion]}$$

where n is the number of charges in the polymer P, $[P^n]$ the concentration of the polymer and [anion] the concentration of the other membrane permeable small anions in the polymer solution. At concentrations of 0.25 mg/ml, there are 0.6 meq of hyaluronan, 0.96 meq of chondroitin sulphate and 1.46 meq of heparin in solution. At physiological anion concentrations of 150 mM, the Donnan coefficients are 1.004, 1.006 and 1.01 for hyaluronan, chondroitin sulphate and heparin, respectively. These are the theoretical values of small ion distributions induced by the presence of 0.25 mg/ml glycosaminoglycans in the culture medium. It is thus impossible to explain a 2.5-fold increase of intracellular pH solely by glycosaminoglycans in solution.

Similar consideration also applies for the decrease of the membrane potential by exogenous glycosaminoglycans [Hagenfeld et al., 2010]. Hyaluronan at a concentration of 0.5 mg/ml depolarised the membrane potential of fibroblasts from -17 to -7.5 mV. The Donnan coefficient is $r_D = 1.008$ at this concentration, and the theoretical potential shift can be calculated from a modified Nernst equation at 37°C

$$\Delta\Phi = \frac{RT}{F} * \ln r_D = 0.2 \text{ mV}$$

Thus the measured potential shift exceeded again the theoretical value. Therefore, we analysed the contributions of receptor binding versus hydrophobic and ionic adsorption in greater detail.

MATERIALS AND METHODS

MATERIALS

Bacterially derived hyaluronan with an average molecular weight of 1.4 10^6 Da [Shiedlin et al., 2004], chondroitin-6-sulphate (Na^+-salt) from shark cartilage, heparin (Na^+-salt) from bovine intestinal mucosa, neuraminidase from *Clostridium perfringens* and other chemicals were from Sigma Chemical Co. Hyaluronan oligosaccharides were prepared as described [Kathagen and Prehm, 2013].

CELLS AND CELL CULTURE

Primary human skin fibroblasts from one donor [Cantz et al., 1972; Hagenfeld et al., 2012] and epithelial HEK-293 cells were grown in Dulbecco's medium supplemented with streptomycin/penicillin (100 units of each/ml), 10% foetal calf serum.

LABELLING OF GLYCOSAMINOGLYCANS WITH FLUORESCEIN

Hyaluronan, chondroitin sulphate and heparin were labelled with fluorescein as described [de Belder and Wik, 1975]. Briefly, the glycosaminoglycans (50 mg) were dissolved in a mixture of 40 ml of water and 20 ml of DMSO. To these solutions acetaldehyde (25 µl), cyclohexylisocyanide (25 µl) and fluorescamine (25 mg dissolved in DMSO) were added and kept at room temperature for 5 h. The reaction mixtures were poured into a mixture of 800 ml ethanol and 2 ml of saturated NaCl. After the precipitated glycosaminoglycans had sedimented, the solution was decanted, the glycosaminoglycans were sedimented by centrifugation for 5 min at 3,000*g* and dried in vacuum.

ADSORPTION OF FLUORESCENT GLYCOSAMINOGLYCANS TO CELLS

Cells were grown on 96-well microtiter plates to confluence which was 3×10^5 cells/cm^2 for fibroblasts or HEK-293 cells and washed twice with normal HEPES buffer (Na-buffer: 136 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5.55 mM glucose, 5 mM HEPES, pH 7.2) or potassium substituted HEPES buffer (K-buffer: 136 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM KH_2PO_4, 5.55 mM glucose, 5 mM HEPES, pH 7.2). The cells were incubated for 10 min with serial 1:1 dilutions of the individual fluorescent glycosaminoglycans starting with concentrations of 2 mg/ml. The solutions were casted off and the cells were washed four times with a solution of 260 mM sucrose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5.55 mM glucose, 5 mM HEPES, pH 7.20 (ISO-buffer). The adsorbed fluorescence was measured by a Synergy HT ELISA reader at the bottom layer at an excitation of 490 nm and emission of 530 nm. The fluorescence intensity was calibrated with the individual fluorescent glycosaminoglycans.

DEPENDENCY OF INTRACELLULAR ACIDIFICATION ON MEMBRANE POTENTIAL

The method and its calibration were described previously [Kathagen and Prehm, 2013]. The membrane potential was annihilated by replacement of Na-buffer by K-buffer or by valinomycin at a concentration of 6.25 µM as shown previously [Hagenfeld et al., 2012]. Cells were grown in 96-well microtiter plates to near confluency. They were washed with HEPES buffer and loaded with fluorescent pH indicator by incubation with 100 µl of 4 µM BCECF-AM for 30 min in HEPES buffer solution at 37°C. The medium was removed, the cells were washed twice with HEPES buffer and incubated with various concentrations of glycosaminoglycans dissolved in Na-buffer or K-buffer. The glycosaminoglycans did not change the pH of 7.2. After 10 min of incubation, the fluorescence intensity was determined in a Synergy HT ELISA reader at an excitation of 490 nm and emission of 530 nm at the bottom of the microtiter plates and the intracellular pH was calculated using a calibration curve.

VISUALISATION GLYCOSAMINOGLYCAN ADSORPTION BY FLUORESCENT MICROSCOPY

Fibroblasts were grown on 10 mm round cover-slips (Gerhard Menzel GmbH, Braunschweig, Germany) in a 24-well microtiter plate to near confluency. Washing procedures were performed as stated above. Briefly, cells were incubated after washing with Na- and K-buffer for 10 min at 4°C. Fluorescent glycosaminoglycans were washed off four times with ISO-buffer on ice. Afterwards cells were fixated with Histochoice MB for 10 minutes and nuclear staining was performed with HOECHST 33342 (Life Technologies, Darmstadt, Germany) at a concentration of 0.1 μg/ml for 10 min. Cover slips were mounted with ROTI Mount Aqua (Carl Roth GmbH, Karlsruhe, Germany) on glass slides (Gerhard Menzel GmbH). Fluorescence microscopy was performed with a LEICA TCS SP5 confocal microscope at a magnification of 1:40. Cells which have been incubated with Na-buffer were adjusted for fluorescence gain. Afterwards K-buffered cells were scanned using a 405 Diode UV and an Argon laser with the same preferences. This procedure was repeated for each individual GAG. The emission spectrum was 417–480 nm for the blue channel and 500 and 580 nm for the green channel. Mean grey-value measurements for each slide were performed with ImageJ.

ADSORPTION OF GLYCOSAMINOGLYCANS AT A GOLD ELECTRODE

An $8 \times 8\,cm^2$ gold foil of 50 μm thickness (24 carat; Dukatshop, Berlin, Germany) was attached to a sticky tape and cut into rectangular strips of $1 \times 2\,cm^2$. A gold-coated strip was immersed into a stirred 10 ml solution of glycosaminoglycan (2 mg/ml) in 66.3 mM potassium phosphate buffer pH 7.2 together with a counter electrode and a reference electrode for 2 min at a current of 0.01 mA and varying potentials from 0 to 800 mV. Potentials were applied using a Wenking potentiostat MP04 (Bank Elektronik, Pohlheim, Germany). The substrate (i.e., the $1 \times 2\,cm^2$ gold-coated tape) served as the working electrode, another gold-coated tape was used as the counter electrode, and an Ag/AgCl (in 3 M KCl) electrode was used as reference. The strip was removed under open circuit conditions and residual solution was removed on a paper towel. The strip was washed for 5 s in a stirred solution of phosphate-buffered saline pH 7.2 and transferred to an Eppendorf tube. Attached fluorescent glycosaminoglycans were desorbed by vortexing with a solution of 1 ml 4 M guanidinium HCl and the amount was determined by a fluorescent spectrometer (Kontron SMF25) at an excitation of 484 nm and emission of 524 nm. The spectrometer was calibrated with fluorescent glycosaminoglycan solutions.

RESULTS

COMPARISON OF GLYCOSAMINOGLYCAN ADSORPTION TO FIBROBLASTS AND HEK-293 CELLS

Adsorption glycosaminoglycans were analysed with hyaluronan-producing fibroblasts which retain an extensive hyaluronan coat on the cell surface by binding to the CD44 receptor, and with hyaluronan- and CD44-deficient human embryonic kidney cells (HEK-293) [Joerges et al., 2012]. All three glycosaminoglycans adsorbed more to polarised fibroblasts (Fig. 1A–C). Figure 2A–C shows the adsorption to HEK-293 cells. The features of the adsorption resemble the above fibroblasts adsorption, although at a lower level suggesting that the CD44 receptor contributed to adsorption.

INFLUENCE OF MEMBRANE POTENTIAL ON GLYCOSAMINOGLYCAN ADSORPTION

The membrane potential was annihilated by substituting the NaCl-buffer by KCl-buffer of equal molarity. This led to reduced adsorption of all three glycosaminoglycans to fibroblasts (Fig. 1A–C) as well as to HEK-293 cells (Fig. 1A–C). Figure 1D shows a steady increase of membrane potential specific adsorption with hyaluronan concentration, whereas chondroitin sulphate and heparin reached saturation. From these data the contribution of membrane potential specific adsorption was calculated for fibroblasts (Fig. 1D) and for HEK-293 cells (Fig. 2D). The results show that the potential-dependent adsorption was greater for fibroblast than for HEK-293 cells. Its proportion remained relatively constant at glycosaminoglycan concentrations between 0.25 and 2.0 mg/ml except for hyaluronan on fibroblasts which showed a steady increase.

It may be possible that the adsorption of glycosaminoglycans are influenced by the endogenous negatively charged neuraminic acids present on cell surface glycoproteins that might partially neutralise the cationic surface layer. Therefore, we digested fibroblasts and HEK-293 cells with neuraminidase prior to adsorption of fluorescent glycosaminoglycans. However, adsorption was unaffected (data not shown).

FLUORESCENCE MICROSCOPY

Since the membrane potential dependent glycosaminoglycan adsorption was larger for fibroblasts, we used these cells to visualise binding. Adsorption was performed at 4°C to minimise endocytosis in normal Na-buffer or in K-buffer which caused membrane depolarisation. Figure 3 shows that depolarised fibroblasts bound all three glycosaminoglycans to a lower extent. The fluorescence intensity was quantified by ImageJ software and shown in Table I.

DEPENDENCY OF INTRACELLULAR ACIDIFICATION ON MEMBRANE POTENTIAL

We analysed, whether alterations of the membrane potential influenced the decrease of intracellular pH by glycosaminoglycans. The membrane potential was annihilated by replacement of Na^+ by K^+ or by the presence of the ionophore valinomycin in the buffer solution as shown previously [Hagenfeld et al., 2012]. Figure 4 shows that glycosaminoglycans did not acidify intracellular pH in the absence of a membrane potential. On the contrary, the intracellular pH even increased above the value of polarised cells. This result suggests that membrane potential-dependent adsorption of glycosaminoglycans is responsible for intracellular acidification.

ADSORPTION OF GLYCOSAMINOGLYCANS AT A GOLD ELECTRODE

To eliminate any cellular factors for adsorption of glycosaminoglycans, we measured the amount of fluorescent glycosaminoglycans adsorbed to an inert gold foil at different surface potentials. Initial experiments indicated that phosphate-buffered saline was

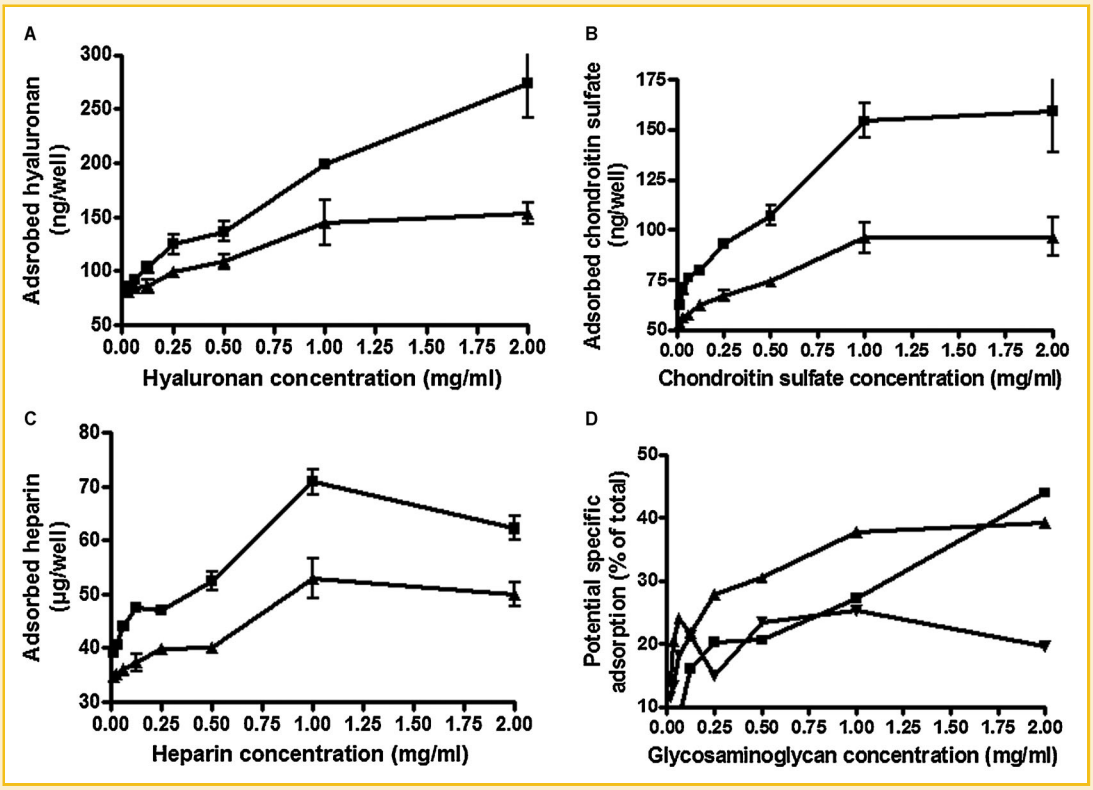

Fig. 1. Glycosaminoglycan adsorption to fibroblasts. Human skin fibroblasts were incubated with increasing concentrations of fluorescent hyaluronan (A), chondroitin sulphate (B) or heparin (C) in NaCl-buffer (■) and KCl-buffer (▲) annihilating the membrane potential. Unadsorbed glycosaminoglycans were washed off and amount of cell-bound glycosaminoglycans was determined. The error bars indicated the SD of three determinations. The percentage of membrane potential-dependent adsorption of hyaluronan (■), chondroitin sulphate (▲) and heparin (▼) was calculated from these data (D).

inappropriate as solvent, because the presence of chloride ions led to electrolysis. Therefore, the glycosaminoglycans were dissolved in 66.3 mM potassium phosphate buffer pH 7.2 which reflected the intracellular physiological ion concentrations. The potential on the gold foil was formed by a potentiometer. Previous studies showed that a surface potential created on a gold foil was in the physiological range between −30 and +120 mV, when the externally applied potential varied between 0 and +800 mV [Barten et al., 2003; Kleijn et al., 2004]. Figure 5 shows that fluorescent hyaluronan indeed adsorbed the charged gold surface depending on the surface potential. Adsorption of fluorescent hyaluronan oligosaccharides, heparin or chondroitin sulphate could not be detected under these conditions (data not shown).

DISCUSSION

The discrepancy between the theoretical and experimental values for the Donnan effect led us to examine the hypothesis that glycosaminoglycans were concentrated by adsorption to the cell surface. Indeed, fluorescent hyaluronan, chondroitin sulphate and heparin adsorbed to CD44 receptor expressing fibroblasts and to a lower extent to CD44 deficient HEK cells, indicating an enhancing effect of CD44. Annihilation of the membrane potential reduced glycosaminoglycan binding, but a basal level of concentration-dependent adsorption remained even in HEK cells. The residual adsorption could be hydrophobic interactions or hydrogen bridges with cell surface components [Scott et al., 2003] and ionic interactions with cell surface proteins [Angello and Hauschka, 1980; Santini et al., 1998; Annabi et al., 2004; Lord et al., 2009]. Adsorption of glycosaminoglycans occurred in two cell types suggesting that it is a general phenomenon independent of cell-specific parameters such as surface area and receptor specificities. Substitution of NaCl buffer with KCl buffer is a physiological method to annihilate the membrane potential and analyse the consequences.

The difference in adsorption could be due to different glycosaminoglycan conformations in NaCl or KCl solutions. This was studied in detail for hyaluronan by X-ray diffraction of crystalline preparations and by light scattering and sedimentation rates and viscosities in solution [Sheehan and Atkins, 1983; Sheehan et al., 1983]. The axial rise of disaccharide units increased from 0.84 nm in NaCl to 0.90 nm in KCl solution. Potassium hyaluronan required more water molecules for stabilisation than sodium hyaluronan and could thus be more protected from interaction.

Adsorption of hyaluronan to a charged gold surface in physiological buffer was also dependent on the surface potential indicating that the surface potential alone was sufficient for binding.

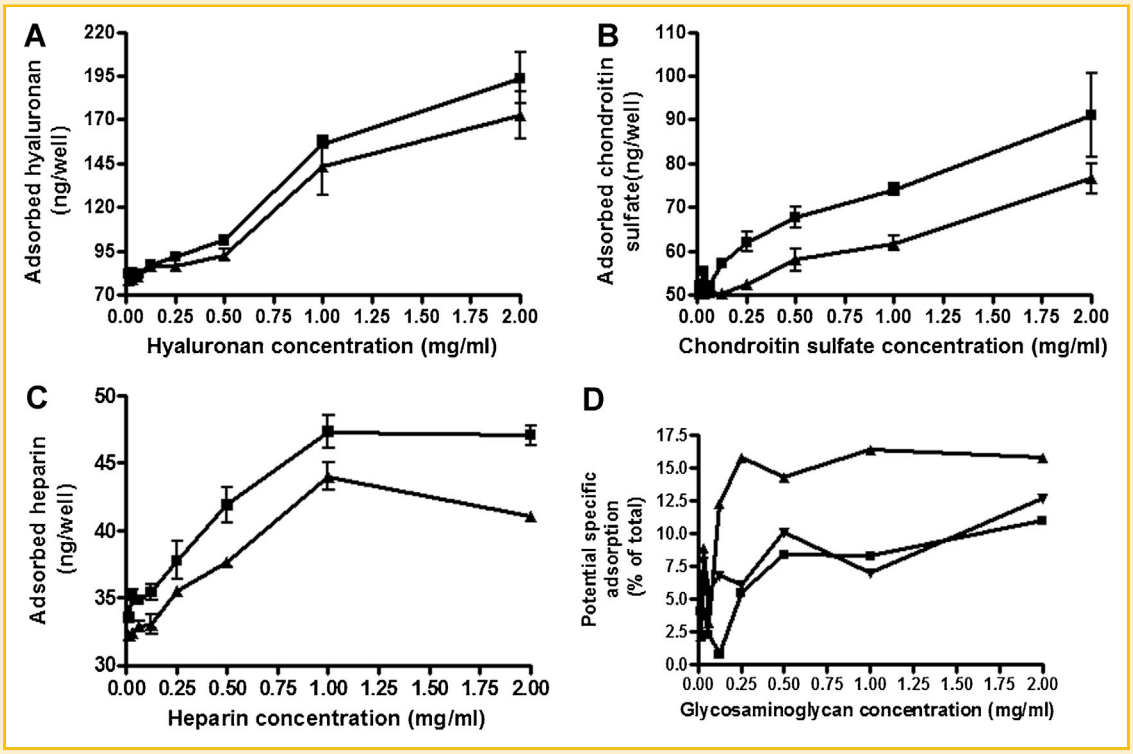

Fig. 2. Glycosaminoglycan adsorption to HEK-293 cells. HEK-293 cells were incubated with increasing concentrations of fluorescent hyaluronan (A), chondroitin sulphate (B) or heparin (C) in NaCl-buffer (■) which maintained and KCl-Buffer as described in Figure 2. The percentage of membrane potential-dependent adsorption of hyaluronan (■), chondroitin sulphate (▲) and heparin (▼) was calculated from these data (D).

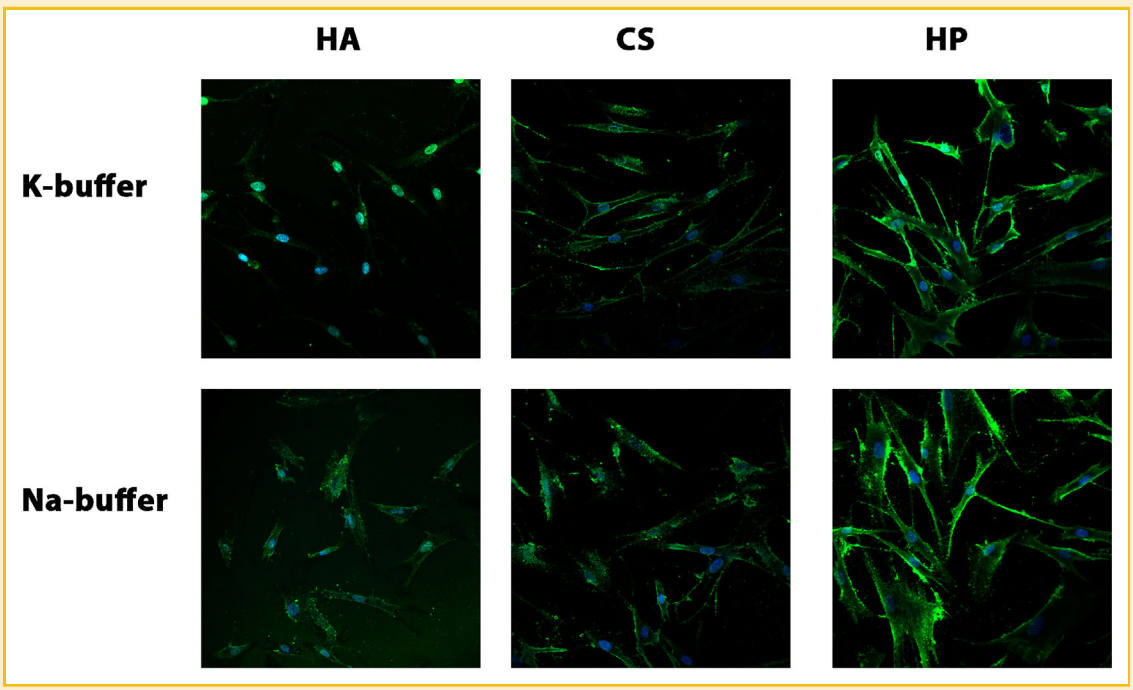

Fig. 3. Fluorescence microscopy of glycosaminoglycan adsorption to fibroblasts. Fibroblasts were incubated at 4°C with fluorescent hyaluronan (HA), chondroitin sulphate (CS) or heparin (HP) in NaCl-buffer (Na) and KCl-buffer (K) annihilating the membrane potential. Blue cell nuclei were stained with HOECHST 33342. Unadsorbed glycosaminoglycans were washed off and fluorescence was visualised by confocal microscopy.

TABLE I. Mean Grey-Value Intensity for Microscope-Pictures Taken From Glycosaminoglycans Incubated With Na- and K-Buffers

	Hyaluronan	Chondroitin sulphate	Heparan sulphate
Na-buffer	17.23	7.40	20.94
K-buffer	7.62	5.96	15.90

In our experiments, adsorption to the gold surface could only be shown for fluorescent hyaluronan, but not for chondroitin sulphate or heparin. Assuming cooperative binding of charges, the affinity should increase exponentially with the number of charges per molecule which is 3818 for hyaluronan, 169 for chondroitin sulphate and 88 for heparin. Thus hyaluronan should have the highest affinity for a charged surface, and this was reflected in our experiment. The fact that hyaluronan adsorbed to the inert gold foil depending on surface potentials similar to those of living cells proved the general phenomenon of ionic adsorption independent of all other cellular parameters such as receptors, buffer composition and hydrophobic interactions.

We have previously shown that hyaluronan addition to cells depolarised the normal resting potential of −17 mV for fibroblasts and −30 mV for HEK-293 cells [Hagenfeld et al., 2010]. This depolarisation must decrease the potassium outflow that is responsible for the formation of the membrane potential. On the other hand, extrusion of hyaluronan through the plasma membrane onto the extracellular matrix is in turn dependent on potassium efflux [Hagenfeld et al., 2012]. The extruding hyaluronan should displace most small anionic counterions such as chloride, because cooperative binding of multiple charges with the polyanion should potentiate the binding affinity as illustrated in Figure 6.

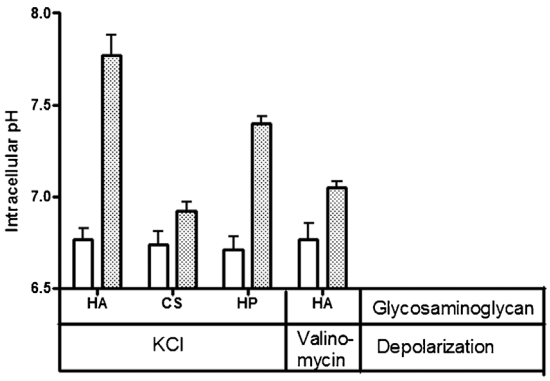

Fig. 4. Dependency of intracellular acidification on membrane potential. Human skin fibroblasts were grown to near confluency. The cells were loaded with the pH indicator BCECF-AM for 30 min, washed and incubated with 0.25 mg/ml of hyaluronan (HA), chondroitin sulphate (CS) and heparin (HP) in normal HEPES buffer (open bars) or HEPES buffer in which NaCl was replaced by equal concentrations of KCl (shadowed bars) to annihilate the membrane potential. Hyaluronan was also added in normal HEPES buffer in the absence (open bars) and presence (shadowed bars) of 6.25 μM of valinomycin. The error bars indicate the SD of six determinations.

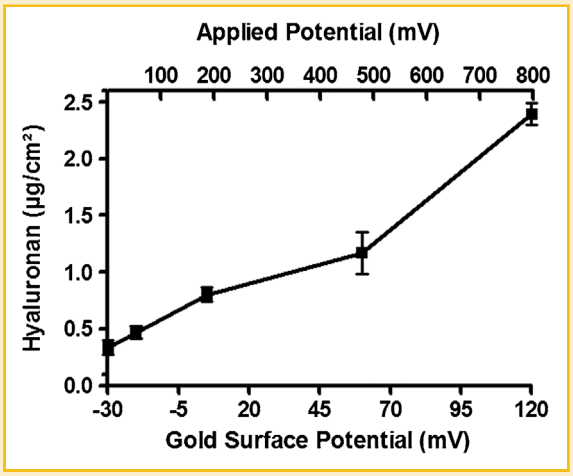

Fig. 5. Adsorption of hyaluronan onto a charged gold foil. A gold foil was immersed into a solution of 2 mg/ml fluorescent hyaluronan in 66.3 mM potassium phosphate pH 7.2 for 2 min at the indicated electric potentials. The corresponding potentials on the gold surface were taken from the published measurements [Barten et al., 2003; Kleijn et al., 2004]. Adsorbed hyaluronan was determined by a fluorimeter at an excitation of 484 nm and emission of 524 nm. The data represent the SD of three determinations.

If glycosaminoglycans adsorb to the cell surface, their Donnan effect expels other ions from their territory and relative changes in macromolecular and small ion concentration (e.g., 150 mM NaCl) can be estimated from the observed difference in intracellular pH by the above equation. A 2.5-fold increase in intracellular H^+ concentration correlates to a 1.5-fold higher concentration of glycosaminoglycan ions than Cl^-. Although it is not possible to calculate the absolute effective glycosaminoglycan concentration in the vicinity of the cell surface, we must assume that such concentration of glycosaminoglycans on the cell surface causes molecular crowding where macromolecules share solvating water [Ryan et al., 1988]. This lack of water would force other ions into lacunae of residual water and make them appear more concentrated [Zimmermann and Minton, 1993].

In conclusion, local glycosaminoglycans concentration led to Donnan effects that exceed their concentration in solution causing depolarisation of the membrane potential [Hagenfeld et al., 2010], cell swelling [Joerges et al., 2012] and intracellular acidification [Kathagen and Prehm, 2013].

Although membrane potential-dependent adsorption of glycosaminoglycans to the cell surface has not been described previously, it is not a surprising phenomenon, because adsorption to cationic detergents, particularly cetylpyridinium chloride, is a well-documented procedure of glycosaminoglycan precipitation and purification [Scott, 1955, 1961, 1962; Laurent and Scott, 1964]. It is interesting to note that the critical salt concentration for adsorption and precipitation of glycosaminoglycans with cationic detergents falls into the range of physiological conditions. Other examples are binding and purification on ion exchangers [Heinegard and Sommarin, 1987] or assay of hyaluronidase activity by turbidity measurements of hyaluronan–albumin complexes [Dorfman, 1955].

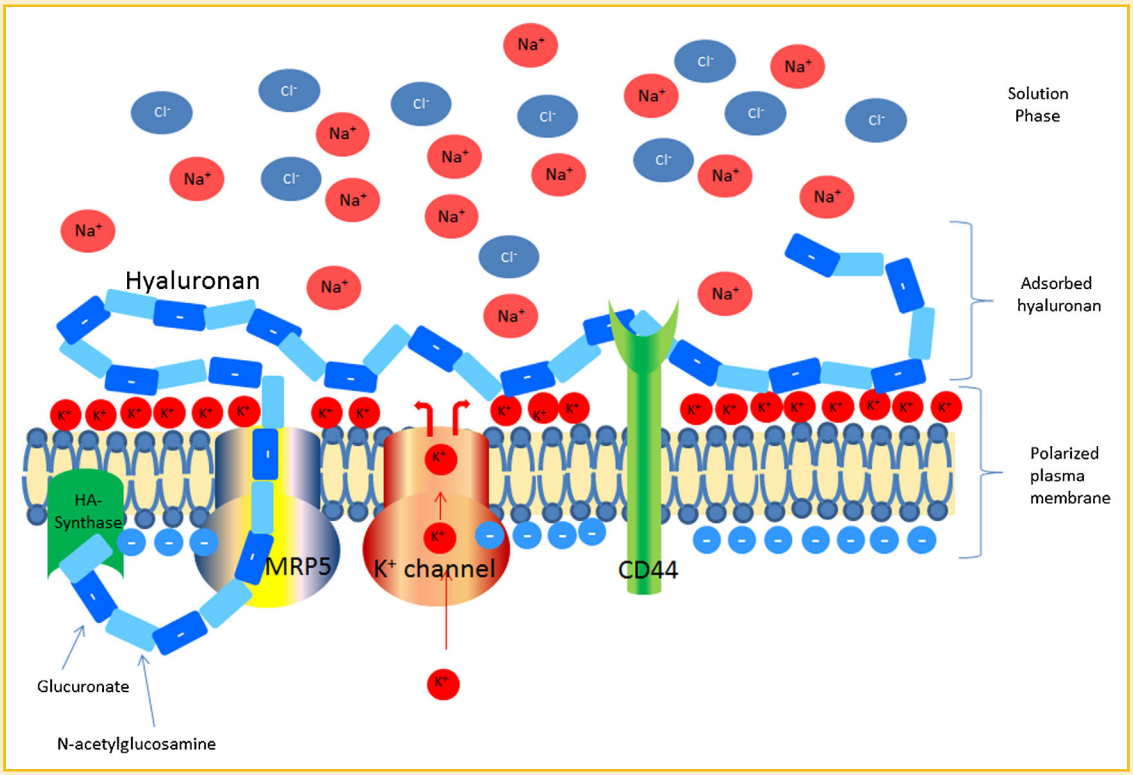

Fig. 6. Model for membrane topology of fibroblasts. Fibroblasts synthesise hyaluronan at the inner side of plasma membranes and export it through the ABC transporter MRP5 concurrently with K^+ to maintain electroneutrality. K^+ efflux is also responsible to create a membrane potential and hyaluronan adsorbs to this layer by cooperative ionic interactions and displaces chloride from this territory. The binding is further stabilised by CD44. This adsorption in the immediate vicinity of the cell surface is responsible for large Donnan effects.

Glycosaminoglycans behave like ion exchangers and impede free diffusion of counterions [Wu, 1926; Maroudas, 1968]. Hyaluronan as a fixed ion exchanger may explain its influence on the electrical impedance of the cerebellum [Wang and Adey, 1969] and other electrical properties of cell membranes [Santini et al., 1998]. It could also serve as the hitherto unknown matrix on the surface of neurons for long-term memory [Kochlamazashvili et al., 2010] which involves storage of charges upon electrical stimulation and local hyperpolarisation, that is, potassium efflux, and subsequent delayed influx of calcium ions [Baudry et al., 2011].

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