Hyaluronan and Renal Fluid Handling

Studies during Normal and Pathological Conditions of Renal Function

BY

VIKTORIA GÖRANSSON
Dissertation for the Degree of Doctor of Philosophy (Faculty of Medicine) in Physiology presented at Uppsala University in 2001

ABSTRACT


The kidney is the major organ responsible for the regulation of the composition and volume of the body fluids, which is essential for homeostasis. The glycosaminoglycan hyaluronan (HA), with extreme water-binding capacity, is present in the interstitium of the kidney with a heterogenous distribution. The importance of HA in renal water-handling is unknown and was the focus of the present investigation.

Acute water-loading in rats caused the amount of papillary HA to increase and during water deprivation, the amount was reduced. Gerbils, with extreme urine concentrating capacity, have less HA in the renal papilla in normal conditions and responded diametrically different to water-loading (reduction in HA). Renomedullary interstitial cells (RMICs), which are probably the main producers of HA in the renal medulla, were cultured at different media osmolalities to mimic the milieu of the medulla during variations in the water balance. The amount of HA found in the media was decreased at high osmolalities and increased at low osmolalities, thereby strengthening the in vivo results. CD44, an HA-receptor involved in the uptake and degradation of HA, was expressed on RMICs in an osmolality dependent manner. During high media osmolality, the CD44 expression increased and at lower osmolalities, the opposite occurred, probably due to the need for uptake and degradation of HA.

Renal ischemia-reperfusion injury causes a cortical accumulation of HA, up-regulation of CD44, and a depression of functional parameters. The time periods of ischemia correlated with the accumulation of HA which, in turn, was inversely correlated to GFR. Hyaluronidase injections in this setting failed to reduce HA levels and significantly improve renal function.

In conclusion, the results from the present study suggest an important role for HA and RMICs in renal water-handling and that the intrarenal distribution of HA is altered after ischemia-reperfusion injury, which correlates with renal dysfunction.

Key words: CD44, diuresis, gerbils, glycosaminoglycan, hyaluronidase, hyaluronan, interstitium, ischemia-reperfusion, kidney, osmolality, oxygen tension, papilla, rat, renomedullary interstitial cells, water.

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Kidney International 58:2061-2068

II **Renomedullary interstitial cells in culture; the osmolality and oxygen tension influence the extracellular amounts of hyaluronan and cellular expression of CD44.**
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III **Renomedullary and intestinal hyaluronan content during body water excess. A comparative study in rats and gerbils.**
Göransson V, Johnsson C, Nylander O and Hansell P, 2001
Manuscript

IV **Renal cortical hyaluronan accumulation after ischemia-reperfusion injury.**
Göransson V, Johnsson C, Häggren R and Hansell P, 2001
Manuscript

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<tr>
<td>ADH</td>
<td>Anti-diuretic hormone, vasopressin</td>
</tr>
<tr>
<td>bw</td>
<td>Body weight</td>
</tr>
<tr>
<td>dwt</td>
<td>Dry weight</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HAS</td>
<td>Hyaluronan synthase</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>PAH</td>
<td>Para-aminomhippuric acid</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>RMIC</td>
<td>Renomedullary interstitial cells</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley rat</td>
</tr>
<tr>
<td>U_osm</td>
<td>Urinary osmolality</td>
</tr>
<tr>
<td>V</td>
<td>Urinary flow</td>
</tr>
</tbody>
</table>
INTRODUCTION

The kidneys are the major organs responsible for maintaining a relatively constant volume and a stable composition of the body fluids, which is essential for homeostasis. By controlling the rate at which the kidney excretes water and electrolytes, a fine balance between input and output can be achieved. The intake of water and electrolytes is usually governed by a person’s eating and drinking habits, forcing the kidneys to adjust their excretion rates to match the intake. This is achieved by several mechanisms involving nerves and hormones and also by intrarenal mechanisms and structural components (Guyton & Hall 1996).

Sympathetic nerves innervate the arterioles and parts of the nephron (Kopp & DiBona 1996). An increase in the sympathetic nerve activity causes contraction of primarily the arterioles leading to a reduced glomerular filtration rate (GFR) and a subsequent increased tubular reabsorption of water and electrolytes. Furthermore, there is also an increase in renin release from afferent arterioles leading to increased plasma levels of angiotensin II and aldosterone and thereby increased reabsorption.

Several hormones influence the renal excretion rate. These hormones can be divided in two groups, anti-diuretic or diuretic, based on their effects on renal water and electrolyte handling. The anti-diuretic hormones include the renin angiotensin aldosterone system, norepinephrine and vasopressin, ADH. ADH is the most powerful hormone for altering the renal excretion of water, independently of the rate of solute excretion. It acts by increasing the water permeability of the distal tubules and collecting ducts, thereby allowing a greater water reabsorption by these segments of the nephron. The water permeability is due to the localization of water channels, so-called aquaporins, to the cell membrane (Nielsen et al 1993).

The diuretic/natriuretic hormones include atrial natriuretic peptide, dopamine and oxytocin. Nitric oxide may also be included in this group.

Intrarenal mechanisms include autoregulation of GFR and renal blood flow (RBF). GFR and RBF are held almost constant over a wide range of arterial blood pressure (70-180 mmHg). Two mechanisms are primarily responsible for the autoregulation of GFR (Guyton & Hall 1996):
1) the myogenic response, which is the ability of vascular smooth muscle cells to spontaneously constrict in response to increased pressure and
2) the tubuloglomerular feed-back, which is the negative feed-back system that responds with a constriction of the afferent arteriole when the GFR is increased.

In the kidneys, water and electrolytes can be reabsorbed all along the nephron, the largest amount (80%) is reabsorbed in the proximal part. The fine-tuning of the excreted amount occurs in the distal parts of the nephron. In these parts, the water permeability of the tubular wall is limited and regulated by ADH. Through the medullary interstitial concentration gradient, water can be reabsorbed from the distal parts of the nephron in the presence of ADH.

The basic requirements for forming concentrated urine are:

I: a high level of ADH in plasma

II: a high osmolality of the interstitial fluid of the renal medulla, providing the osmotic gradient necessary for water reabsorption seen in the presence of ADH.

Structural components may also be important for the fluid balance. For example, the interstitium of the renal medulla contains fibroblasts and matrix components which can, directly or indirectly, influence fluid transport. One such matrix component is hyaluronan, HA. The renal medulla contains very high amounts of HA, while the cortex is virtually void of this substance in normal physiological conditions. This distribution is altered in renal diseases. The physico-chemical properties of HA may modulate diffusion processes in the medulla, an effect which may be due to the water binding properties of HA and its high resistance to fluid flow. Due to the unique intrarenal distribution of HA and its unique water binding properties, HA might be expected to have a large influence on the renal handling of water, a hypothesis which was elucidated in the present study.
Hyaluronan

HA is a large, linear, negatively charged structural component, belonging to the family of glycosaminoglycans. It was first discovered in the vitreous humour by Meyer and Palmer in 1934 (Meyer & Palmer 1934). They named it hyaluronic acid after the site where it was found (hyaloid=glassy), and its content of uronic acid. In the mid 1980’s, it was renamed hyaluronan in accordance with the modern biochemical nomenclature of polysaccharides (Balazs et al 1985) since it exists as a polyanion and not as an acid in vivo. HA was first believed to only have a role as a space-filling and lubricating molecule, but today it is known to have specific effects on cell function and to be of importance for a variety of biological functions (Comper & Laurent 1978, Scott 1989, Laurent & Fraser 1992). The molecular functions of HA are today considered to fall into three partially overlapping categories: First, HA occupies an enormous hydrodynamic domain that influences the hydration and physical properties of the tissues. Second, it interacts with other extracellular matrix macromolecules, such as versican and aggrecan; interactions believed to be essential to the structure and assembly of several tissues. Finally, HA interacts with cell surface receptors, notably CD44, and thereby influences cell behavior (Toole 2000).

Structure and properties of HA

Determining the exact chemical structure of HA took quite some time (Weissman & Meyer 1954). The chemical structure is simple, consisting of repeated disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid linked by $\beta(1-4)$ and $\beta(1-3)$ glycosidic bonds, respectively. HA is different from other glycosaminoglycans in that it is neither sulphated nor covalently linked to a protein during synthesis. The negative charge of the molecule is due to ionisation of the carboxyl groups of the glucuronic acid constituents at physiological pH.

The length of the molecule can vary, ranging from just a few disaccharides up to several thousand, giving the molecule different properties. When the amount of HA in a solution reaches a concentration of approximately 0.1 mg/ml, the molecule can form an entangled network. Inter- and intra-molecular interactions occur such that the volume occupied by HA is increased, resulting in the exclusion of other macromolecules from their normal molecular environment. This phenomenon, called
steric exclusion, can influence both osmotic activity and water transport in the extracellular matrix since water is immobilized and flow restricted (Comper & Laurent 1978, Laurent & Fraser 1992).

**Turnover of HA**

*Synthesis*

In 1983, Prehm found that HA synthase (HAS) was present in the plasma membrane and that the synthesis of HA occurs in the inner part of the plasma membrane. The newly synthesised HA is extruded into the extracellular space. This extrusion mechanism results in very large polymers, something that could not occur if the molecule were synthesized in the Golgi apparatus within the cell (Prehm et al 1983, Philipson & Stewart 1984). The HAS exists in several different isoforms, where HAS1, HAS2 and HAS3 have been identified in vertebrates so far (Spicer et al 1996, Weigel et al 1997, DeAngelis 1999). HA synthesis is regulated by various factors in mammals, such as growth factors, hormones and inflammatory mediators (Heldin et al 1989, Spicer et al 1996, Jacobson et al 2000).

*Catabolism*

There is a rapid turnover of HA in the body. About one third of the total content is turned over daily (Laurent & Fraser 1986, Laurent & Fraser 1992). The half-life for a molecule in the blood is very short, only a few minutes. The degradation takes place in central organs, such as the liver, spleen and kidney, which take up HA from the blood. HA can also be degraded locally in other tissues such as joints and muscles (Fraser & Laurent 1989, Smedrød 1991). Hyaluronidases are the enzymes responsible for most of the degradation of HA (Kreil 1995). Several hyaluronidase isoforms exist, Hyal1, Hyal2, Hyal3, Hyal4 and PH-20/Spam-1 (Kreil 1995, Sun et al 1998, Stern & Csóka 2000). Hyal 1-3 has been found in the kidney (Bollet et al 1963, Sun et al 1998). The Hyaluronidase activity is known to be low in the outer parts, i.e. cortex, but increases significantly towards the tip of the papilla (Goryunova et al 1975).

**HA receptors**

Several HA binding proteins are known and are usually termed "hyaladherins" or "hyaluronan binding proteins" (Toole 1990, Turley 1991, Day 1999). As mentioned
earlier, some effects of HA include a direct effect on cell behavior. This matrix-cell interaction is mediated via cell-surface matrix receptors. Several receptors for HA have been identified including: CD44, RHAMM (Receptor for HA Mediated Motility) and LEC-receptor (Liver Endothelial Cell clearance receptor). The two most recently described are HARE (HA Receptor for Endocytosis, Zhou *et al* 2000) and a receptor named layilin, believed to play a role in cell adhesion and motility (Bono *et al* 2001). CD44 is the most common and best studied HA receptor (Toole 1990, Underhill 1992, Lesavre *et al* 1997). HA does not only bind to these surface receptors but also, via a link molecule, to proteins in the extracellular matrix. Versican, aggrecan and neurocan are all part of this link protein family, and are usually found in the interstitium and are not attached to the cell surface. The binding of HA to these proteins in the matrix is believed to be more important for the arrangement and modulation of the extracellular matrix rather than having specific effects on cell behavior and HA turnover as compared to cell surface receptors.

**CD44**

The principal cell surface receptor for HA is CD44. It is found on a variety of cells ranging from chondrocytes and keratinocytes to cells of the immune system and several kinds of tumour cells (Aruffo *et al* 1990, Toole 1990, Underhill 1992, Knudson & Knudson 1993, Lesavre *et al* 1997). The receptor is a transmembrane glycoprotein and consists of four functional domains. Modulation of these domains, through alternative splicing, leads to changes of the structure and thereby changes in HA binding as well as CD44 interactions with the cytoskeleton (Toole 1990, Knudson & Knudson 1993, Lesavre *et al* 1997). There are several cellular functions of CD44. The receptor is known to participate in cell-cell aggregation, matrix-cell signalling, cell migration and receptor mediated internalization/degradation of HA (Culty *et al* 1992, Hua *et al* 1993).

**HA and the kidney**

A publication in *Nature* 1958 by Ginetzinsky was the first to mention HA and hyaluronidase in the kidney and the study was the first to show how the amount of hyaluronidase excreted in the urine was altered by the need for water-reabsorption and excretion. The amount of hyaluronidase excreted in the urine was found to increase
after thirst and drop to zero after water loading, probably changing the amount of HA in the papilla accordingly (Ginetzinsky 1958). In the late 1970’s and early 1980’s, Rowen and Law performed a series of experiments where they found that antiserum against rat urinary impaired the concentrating capacity in rats subjected to anti-diuretic stimuli. Therefore, they hypothesized that the activity of renal hyaluronidase is necessary for maximal anti-diuresis and associated osmotic and morphological changes within the renal medulla (Law & Rowen 1981, Rowen & Law 1981). HA in the kidney was later studied in several pathological models, such as renal graft rejection (Hällgren et al 1990a, Wells et al 1990), unilateral uretheral obstruction (Johnsson et al 1997) and warm renal ischemia reperfusion (IR) injury (Johnsson et al 1996, Lewington et al 2000). A heterogenous distribution of HA in the healthy kidney was observed in all these studies. Large amounts of HA were found in the inner parts of the kidney, i.e. the papilla (approximately 400 µg/g dwt), less was found in the outer medulla (approximately 250 µg/g dwt) and very small amounts in the cortex (<5 µg/g dwt) (Hällgren et al 1990a, Johnsson et al 1996, Hansell et al 2000). Under pathological conditions, an accumulation of HA occurred, primarily to the cortex.

HA may be regulated in response to the water balance of the organism. During excessive water intake, the relative water reabsorption needs to be reduced in the medullary collecting duct system. There is also in vitro evidence of an effect of ADH on the hyaluronidase activity. Stimulation by ADH causes increased Hyaluronidase activity (Ginetzinsky 1958, Nikiforvskaia et al 1987, Ivanova & Melidi 1999). The idea is that the presence of HA in the interstitium reduces the movement of water (Laurent & Fraser 1992, Hardingham et al 1999) and also forms a “functional” oedema, separating the structures involved in water reabsorption (Hansell et al 2000). The properties of the HA rich matrix may also involve changes in the interstitial hydrostatic pressure (Zawieja et al 1992). Another theory for the function of HA in the renal interstitium is that the presence of HA stabilizes the cortico-medullary concentration gradient (resists hydraulic flow) needed for medullary water reabsorption.
Ischemia-reperfusion injury

In 1990, it was demonstrated that a local accumulation of HA occurs in the cortex during renal transplant rejection (Hällgren et al 1990a, Wells et al 1990 and 1993). A similar, but slighter, accumulation is observed in the early post-transplantation period, and it was thus speculated that ischemia per se could result in an increased HA content of the tissue. Studies performed in animal models of renal IR injury (Johnsson et al 1996, Lewington et al 2000) suggested that this was the case. The accumulation of cortical HA is paralleled with an increase in the cortical water content, probably due to the unique water binding capacity of HA (Comper & Laurent 1978, Hällgren et al 1990a). Thus, the interstitial oedema known to occur after transplantation (Hällgren et al 1990a), which in turn influences vascular resistance and tubular function, can partly be due to the accumulation of HA.

Gerbils

The major part of this study was performed in rats and on renomedullary interstitial cells. However, the present study also investigates the Mongolian gerbil (a desert rodent, Meriones Unguiculatus). The interest in this species emanates from a number of investigations comparing the rat and the gerbil, showing how these species differ in several respects regarding water handling. As concerns the kidney, there are anatomical and physiological differences (Schmidt-Neilsen 1964, Buchanan & Stewart 1974, Natochin et al 1983). For example, the papilla of the gerbil is much longer than that of the rat, and thus the vasa recta and loops of Henle are longer. This leads to an increased ability to create a concentration gradient, which is needed for the formation of concentrated urine. The rat can concentrate its urine up to approximately 3,000 mOsm/kg H₂O, for the gerbil the corresponding figure is approximately 5,000 mOsm/kg H₂O and for man 1,200 mOsm/kg H₂O. Regarding the renomedullary interstitial cells, RMICs, there are also species-related differences. This cell-type is found in the medulla and their most characteristic feature is the abundance of lipid droplets. They are believed to provide structural support to the renal tubules and blood vessels but also to be involved in the regulation of renal blood flow and urine concentration. In water-loaded rats, which excrete urine of relatively low osmolality, the number of lipid droplets in the RMICs was two times larger than in untreated rats.
In similar studies of gerbils, the amount of lipid droplets decreased after water loading (Bohman & Jensen 1978). The exact role of these lipid droplets is not known but is believed to be involved in the regulation of renal function. An important factor is, naturally, ADH, which translocates aquaporins from intracellular vesicles into the apical membrane of the distal tubule and collecting system (Nielsen et al 1993), leading to an increased ability to reabsorb water. Under control conditions the ADH levels are four times higher in the gerbil as compared to the rat (Huang et al 1994, Baddouri & Quyuo 1991).

**Brattleboro rat**

Some experiments in the present investigation are performed in Brattleboro rats, which is a model of Diabetes Insipidus. The rat is genetically deficient of ADH, resulting in the inability to concentrate its urine, and therefore, this animal produces a large urine volume of low osmolality.
AIMS OF THE INVESTIGATION

The general purpose of this study was to gain more knowledge about the role of HA in renal fluid handling both under physiological and pathological conditions.

Studies were undertaken with the following specific objectives:

- To elucidate the possible involvement of papillary HA in renal water handling.

- To elucidate parameters determining HA turnover in renomedullary interstitial cells in culture.

- To compare the intrarenal distribution and amount of HA between normal rats and gerbils as well as the changes occurring with water loading in the two species.

- To elucidate if a change in the intrarenal distribution of HA, due to ischemia-reperfusion injury, causes alterations in the kidney function and investigate if treatment with the HA degrading enzyme hyaluronidase could improve the kidney function.
MATERIALS AND METHODS

Animals
All experiments on animals were approved by the local ethics committee at Uppsala University or the local ethics committee at University of Melbourne, Australia. Two different species were used in this thesis, rats and gerbils. A total of 147 rats and 51 gerbils were used.

Rats: Mainly male Sprague-Dawley rats were used and they were bred either at M&B, Skensved, Denmark or B&K, Sollentuna, Sweden. The weight of the rats were 277 ± 33 g. Five Brattleboro rats with Diabetes Insipidus (Harlan-Nederland, Horst, The Netherlands) were also used, weight 319 ± 4 g.

Gerbils: Gerbils bred at the Laboratory Animal Department, Biomedical Center, Uppsala, Sweden were also used, weight 76 ± 8 g.

Up to the day of the experiment, the animals had free access to tap water (except animals subjected to dehydration) and a standardized chow (R3, Ewos, Södertälje, Sweden) containing 0.3% sodium, 0.8% potassium and 21% protein. Kidneys for isolating the renomedullary interstitial cells were obtained from young male Sprague-Dawley rats weighing 80-90 g.

Three studies were performed in vivo (Study I, III, IV) and one performed in vitro (Study II). To simplify the presentation, they will be described separately below.

In vivo experiments

Anesthesia
The animals were anesthetized either with an intra peritoneal injection of Inactin® (120 mg/kg body weight (bw), Byk-Gulden, Konstanz, Germany) or by gas-anesthesia with Forene® (Isoflurane, Abbott Scandinavia AB, Kista, Sweden), mixed in 40% O₂ and 60% air. The gas was either delivered through a breathing mask (during implantation of chronic catheter and induction of ischemia, Study IV) or with a ventilator (Model 683, Harvard Apparatus Inc., MA, USA) connected to a tracheal tube (renal function studies, Study IV). The animals were placed on a servo-controlled heating pad to maintain the rectal temperature at 37.5-38.5°C.
**Surgery**

After tracheotomy, polyethylene catheters were inserted into the right femoral vein and artery of some animals, the former for infusion. The arterial catheter was used for continuous measurements of mean arterial blood pressure (MAP) through a pressure transducer, and for blood sampling. For urine sampling, either the urinary bladder was catheterised through a suprapubic incision or the left and right ureter were both cannulated for urine sampling, in *Study IV*. Since the rats were under gas-anesthesia delivered with a ventilator in *Study IV*, blood samples were taken 2-3 times during the experiments to follow acid-base parameters.

In *Study IV*, a chronic catheter was placed in the left jugular vein just before the induction of renal ischemia and then tunneled up under the skin to produce an i.v. channel for daily injections of hyaluronidase (20,000 units/kg bw, Sigma Chemical Co., St. Louis, MO, USA, in 0.2 ml PBS with 20 mg/ml albumin) or vehicle (0.2 ml PBS with 20 mg/ml albumin) during the reperfusion phase (72h).

After the experiments, the kidneys were excised, weighed, sectioned and then frozen and stored in –70°C until analyzed. All animals were euthanized with an intravenous injection of saturated KCl.

**Experimental protocols**

After the completion of the surgical procedures, the animals were stabilized for 60 min and during that time given a continuous infusion of isotonic saline at 0.5 ml/h·100g or 0.15 ml/h·100g bw or hypotonic glucose-saline solution (0.25% NaCl, 0.5% glucose, approximately 100 mOsm/kg H₂O) at an infusion rate of 1.5 ml/h·100g bw (*Studies I and III*, respectively) or an infusion of saline (0.15 ml/h·100g bw) containing ³H-inulin (1.75 μCi bolus followed by 0.5 μCi/h·100g bw; NEN, Boston, Mass., USA) and paraaminohippuric acid (PAH, 4-aminohippuric acid, 2 mg bolus followed by 0.6 mg/h·100g bw; Merck Darmstadt, Germany) (*Study IV*). The animals received different treatments as described below.

*Study I: Hyaluronan content in the kidney in different states of body hydration.*

In this study, the animals were divided into six groups:
1) **Euvolemia**: isotonic saline was infused and after 60 min of equilibration, a 20 min urine collection period started.

2) **Water diuresis**: hypotonic glucose-saline was infused following priming with 15 ml/kg. After 100 min of equilibration, a 20 min urine collection period started.

3) **Water diuresis and desmopressin**: as in group 2) and then immediately thereafter, 2 ng of the vasopressin V₂-receptor agonist desmopressin (Minirin®, Ferring AB, Malmö, Sweden) was given in 2-3 min and urine was sampled for another 20 min period.

4) **Anti-diuresis**: 24 hours prior to the experiments, the animals were deprived of water. No baseline intravenous infusion was given. The bladder was exposed, punctured with a needle and the urine was collected.

5) **Gerbils**: 12 hours prior to the experiments, the animals were deprived of water. No baseline intravenous infusion was given and after 60 min of equilibration, a 20 min urine collection period started.

6) **Brattleboro rats**: isotonic saline was infused and after 20 min of equilibration, a 20 min urine collection period started.

Urine was collected for the analysis of flow rate and urine osmolality. The kidneys were sectioned and analysed for their HA content.

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**Study III: Renomedullary and intestinal hyaluronan content during body water excess. A comparative study in rats and gerbils.**

The animals, rats and gerbils, were divided into four groups,

1) **Control**: isotonic saline solution was infused. Urine was collected during four consecutive 30 min sampling periods.

2) **Water diuresis 2h**: hypotonic glucose-saline solution was infused. Urine was collected during four consecutive 30 min sampling periods.

3) **Water diuresis 4h**: as in group 2) but urine was collected during eight consecutive 30 min sampling periods.

4) **Water diuresis 6h**: as in group 2) but urine was collected during 12 consecutive 30 min sampling periods.
Urine was collected for the analysis of flow rate and urine osmolality. The kidneys were excised, weighed and sectioned for quantitative HA analysis or histochemical staining for HA. In groups 1) and 2), the intestine was excised and sectioned into specimens of duodenum (~1 cm from pylorus), jejunum (~10 cm from pylorus), ileum (~2 cm from the ileocecal valve i.e. distal ileum) and proximal (~3 cm from the ileocecal valve) and distal part of colon (~4 cm from rectum) under microscope at low magnification and then used for quantitative HA analysis.

Study IV: Renal cortical hyaluronan accumulation after renal ischemia-reperfusion injury.

Model of renal ischemia-reperfusion
Under gas-anesthesia, the left kidney was exposed through a midline incision and totally freed from surrounding tissue. The ureter, renal vein and artery were clamped with microvascular clips, thereby blocking inflow and outflow to the kidney. Two minutes before the clamps were removed, the animals received 20,000 units/kg bw hyaluronidase, vehicle or no treatment. After 20, 30 or 45 min the clamps were removed and the abdomen closed with ligatures. After the ischemia had been induced, the animals received daily i.v. injections of either hyaluronidase or vehicle. Other animals received continuous infusions at the time for functional studies. Sham operated rats underwent the same surgical procedure with the exception of clamping the renal vessels and the ureter. Three days (72h) after the induction of ischemia, the rats were anesthetized and renal function studies were performed. The animals were subjected to several different time periods of ischemia and two different treatments as listed below:
Table of the different experimental groups.

<table>
<thead>
<tr>
<th>Group/ ischemic time</th>
<th>n</th>
<th>Treatment</th>
</tr>
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<tbody>
<tr>
<td>0 min Sham, Vehicle</td>
<td>6</td>
<td>Daily injections of vehicle d. 0-3</td>
</tr>
<tr>
<td>0 min Sham, Hyal daily</td>
<td>7</td>
<td>Daily injections of Hyal d. 0-3</td>
</tr>
<tr>
<td>20 min, Vehicle</td>
<td>7</td>
<td>Daily injections of vehicle d. 0-3</td>
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<td>20 min, Hyal daily</td>
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<td>20 min, Hyal cont</td>
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<td>Daily injections of vehicle d. 0-3</td>
</tr>
<tr>
<td>45 min, Hyal daily</td>
<td>6</td>
<td>Daily injections of Hyal d. 0-3</td>
</tr>
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(Hyal=hyaluronidase).

Urine and plasma analysis

The urine volumes were measured gravimetrically. The urinary osmolality ($U_{osm}$) was estimated from the depression of the freezing point (Model 3MO, Advanced Instr Inc., USA). Urinary sodium and potassium concentrations were determined, using flame photometry (FLM3; Radiometer, Copenhagen, Denmark). $^3$H-inulin in samples of plasma and urine was detected using a liquid scintillation counter (PW 4700, Philips, Holland) and PAH through a chemical spectrophotometric assay (Lambda 2, Perkin-Elmer & Co GmbH, Überlingen, Germany). The acid-base status was determined from arterial blood samples (AVL Compact 3, AVL LIST GmbH, Graz, Austria).

Glomerular filtration rate and renal blood flow measurements

Glomerular filtration rate (GFR) and renal plasma flow (RPF) were estimated from the clearance of $^3$H-inulin and PAH, respectively, according to:

$$\text{Clearance of } s = \left( C_s, \text{urine} \cdot V \right) / C_s, \text{plasma}$$  \hspace{1cm} \text{Equation 1}

where $C_s$ is the concentrations of $^3$H-inulin or PAH in urine and plasma, respectively, and $V$ represents the urine flow rate. Renal blood flow (RBF) was calculated according to:
RBF = RPF / (1-Hct)  

Equation 2

where Hct is the systemic hematocrit. Since the extraction of PAH is not complete, there will be an underestimation of RBF if not corrected for. Therefore, measurements of the arteriovenous (a-v) difference for PAH ((aPAH – vPAH)/ aPAH) were made in 3 anesthetized normal rats. The renal artery and vein were cannulated and blood samples (100 µl) obtained after a 60 min equilibration period for PAH analysis. The calculated a-v difference was 0.44. All calculated RBF values (Equation 2) were therefore divided by 0.44. It is known that vascular permeability and transport mechanisms are changed in ischemia-reperfusion injury, and therefore, it is questionable whether the a-v difference used for correction is valid for all groups of animals. The extraction may, furthermore, vary between the different ischemic periods tested. The same line of reasoning can be put forward regarding the use of inulin and PAH as such estimating GFR and RPF in models of renal damage. However, the calculated GFR and RPF values should provide indications of the functional status of the kidney.

Quantitative assay of HA and determination of water content

The sectioning of the kidneys into specimens of cortex, outer medulla and inner medulla (papilla) has previously been described in detail (Karlberg et al 1983). All specimens were put on filter paper for 3 min and then weighed (wet weight). The specimens were lyophilised overnight and weighed again (dry weight). After grinding, HA was extracted from the tissues for 16 hours with 0.5 M NaCl at 4°C. Following centrifugation for 15 min at 2,700 g, the HA content of the supernatants was measured using a commercially available radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden). The technique is based on the binding of HA to specific a HA-binding protein (Tengblad 1980). Briefly, a 100 µl of sample or standard was mixed with 200 µl ¹²⁵I-labeled HA-binding protein and incubated for 60 min at 4-7°C. 100 µl HA-Sepharose at a concentration of 1 mg/ml was added and then the tubes were incubated an additional 45 min at the same temperature. Two ml of washing solution was added and the HA-Sepharose was recovered after centrifugation at 2,000 g for 10 min. Bound reactivity in the pellet was measured with a gamma counter. Each sample was tested in
duplicates. A standard curve was constructed by using known amounts of HA and the radioactivity was plotted as a function of HA concentration. The variability was <10%. The water content of the tissues was calculated as (wet weight - dry weight)/wet weight.

**Histochemical analysis**
The specimens were fixed in 4% buffered formalin, pH 7.3, with 1% cetylpyridinium chloride and stored at room temperature until embedded in paraffin and sectioned. For the detection of HA, an avidin-enzyme, biotin-protein system was used. In brief, the sections were incubated with bovine serum albumin (10 mg/ml, Fraction V, Sigma Chemical, St Louis, USA) to block non-specific binding sites and then in 3% H$_2$O$_2$ in phosphate-buffered saline to inhibit endogenous peroxidase. After an incubation of 2 hours with a specific biotinylated HA-binding protein, the sections were incubated with ABC Vectastain Reagent (Vector Laboratories, Burlingame, CA, USA) for 1 hour. Finally, H$_2$O$_2$ as substrate and 3-amino-9-ethyl-carbazole, AEC, as electron donor were added, whereafter the specimens were counterstained with Mayer’s hematoxylin. Control sections were incubated for 2 hours with Streptomyces hyaluronidase.

To determine the identity of infiltrating cells and examine the distribution of the HA binding receptor CD44, immunohistochemical analyses were also performed using the monoclonal antibodies R73, ED1, OX6 and OX50 (Serotec, Oxford, UK). R73 detects α/β-receptor expressing T-lymphocytes, ED1 macrophages, OX6 MHC class II-expressing cells and OX50 CD44-expressing cells. Specimens for this staining were obtained three days (72h) after 20, 30 and 45 min of left renal ischemia, respectively. The kidneys were excised and immediately frozen in liquid nitrogen and stored at –70°C until cryosectioned. Six µm thick sections were cut on a cryostat at –22°C, and air-dried. The sections were fixed in 100% acetone and, thereafter, incubated in 0.3% H$_2$O$_2$ in phosphate-buffered saline to inhibit endogenous peroxidase. Non-specific binding was blocked with goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and after that, the primary antibody was added and the sections incubated for 30 min. A secondary antibody, IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was added in excess. Then, the
sections were incubated with horseradish peroxidase-mouse antiperoxidase (Dakopatts, Glostrup, Denmark). Finally, H₂O₂ as a substrate and AEC as electron donor were added to react with the horseradish peroxidase. The sections were counterstained with Mayer’s hematoxylin. Negative controls were obtained by omitting the primary antibody. All slides were evaluated blindly.

**In vitro experiments**

**Cell isolation**

Renomedullary interstitial cells (RMICs) were isolated from kidneys of young Sprague-Dawley rats, using a method described by Fontoura et al. (1990) and modified by Maric et al. (1996). The animals were anesthetized with pentobarbitone sodium (Nembutal 40 mg/kg intra peritoneal). After a midline incision, a needle was inserted in the abdominal aorta and the kidneys were retrogradely perfused at 150 mmHg with sterile Hanks’ balanced salt solution, until cleared of blood. The kidneys were then removed and the medullae were dissected. The tissue was finely cut and digested with 0.1% collagenase type I for 30 min at 37°C. Digested fragments were sieved and dispersed cells were resuspended in a 1:1 mixture of culture medium RPMI 1640 and Dulbecco’s modified Eagle’s medium (DMEM), conditioned by 3T3 Swiss albino mouse fibroblasts. Cells were kept at 37°C in a 95% air-5% CO₂ incubator. Homogenous cell populations were usually reached at passage 10 and in subsequent experiments used up to passage 20.

**Experimental protocol**

*Study II: Renomedullary interstitial cells in culture; the osmolality and oxygen tension influence the extracellular amounts of hyaluronan and cellular expression of CD44*

RMICs plated at 2x10⁵ cells/cm² (either in 25 cm² flasks or on glass slides) were grown in an RPMI 1640 culture medium containing 10% fetal bovine serum for 2 or 4 days, reaching sub-confluence or confluence, respectively. The osmolality of the medium and the oxygen tension were adjusted as described below. Following the manipulation of osmolality and oxygen tension:
1. Supernatants were collected for assay of their HA content, whilst the cells were trypsinsated and the final number of cells counted, using a routine hemocytometer method. Cell viability was determined by trypan blue exclusion.

2. Cells were grown on glass slides and CD44 was localised by immunostaining.

3. Cells were grown in 25 cm² flasks and analysed for CD44 by flow cytometry.

**Manipulation of medium osmolality**

The osmolality of the medium was increased daily by increments of 300 mOsm/kg H₂O by the addition of NaCl and mannitol. The final concentrations of NaCl and mannitol at each osmolality were (in M): 630 mOsm/kg H₂O, 0.08 and 0.1; 930 mOsm/kg H₂O, 0.16 and 0.2; 1,230 mOsm/kg H₂O, 0.24 and 0.3, respectively. To achieve an osmolality of 230 mOsm/kg H₂O, the culture medium was diluted 2:3 with distilled water.

**Manipulation of O₂ tension**

RMICs were grown under either normoxic (95% air/5% CO₂, giving the final concentration of 20% O₂) or hypoxic (1% O₂/5% CO₂/94% N₂ or 5% O₂/5% CO₂/90% N₂) conditions for 2 or 4 days.

**Measurement of HA in supernatants**

The HA content in the supernatants was measured in duplicates, using the same radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden) as described under “*In vivo* experiments” above.

**Measurement of CD44 immunoreactivity**

*Immuno-localisation:* Following the fixation with Bouvin’s fixative for 2 hours at room temperature, the cells were washed with phosphate buffered saline (PBS) and the endogenous peroxidase was blocked with 4% H₂O₂ in methanol for 20 min. After incubation with goat/rabbit serum for 20 min at room temperature, the cells were incubated with the primary antibody (CD44, Santa Cruz, Santa Cruz, CA, USA) overnight at 4°C and then washed. A secondary biotinylated antibody was then applied at room temperature for 1 hour. The specific immune reaction was amplified by
applying the avidin-biotin complex (ABC Vectastain, CA, USA) for 1 hour and the reaction was visualised with DAB (3,3′-diaminobenzidine tetrahydrochloride dihydrate). The cells were then counter-stained with Mayer’s hematoxylin, dehydrated with ethanol and mounted in DPX (Dako, Denmark).

**Flow cytometry:** Following the fixation with 4% paraformaldehyde at room temperature for 2 hours, the cells were washed with PBS, scraped with a rubber policeman and centrifuged at 1,000 rpm for 5 min. Then, the cell pellet was incubated in goat serum (negative control), IgG (isotype control) or CD44 antibody for 2 hours. Cell suspensions were washed twice with PBS and then incubated with FITC-labelled goat antiserum. After a 4-hour incubation, the cells were washed twice with PBS and the fluorescence was measured using a FACS Calibur (Becton & Dickinson Immunocytometry Systems, San Jose, CA, USA). A minimum of 10,000 events was acquired for each sample. The mean fluorescence intensity (MFI) on all events was determined as MFI_{test sample}-MFI_{negative control}, and expressed as a percentage change from the control (330 mOsm/kg H₂O or 20% O₂).

**Statistical analysis**

All data are presented as means ± standard errors of the mean (SEM). Comparisons between groups have been performed with Student’s unpaired t-test or linear regression, *Study I*. A one- or two-way analysis of variance, ANOVA, with the multiple-comparison Tukey test was used in *Study II*. In *Studies III and IV*, Student’s unpaired t-test or ANOVA followed by Fisher’s PLSD post hoc test were used when appropriate. The correlation between HA and GFR was analysed by linear regression, *Study IV*. A P-value of less than 0.05 was considered to be statistically significant.
RESULTS AND COMMENTS

I. Hyaluronan content in the kidney in different states of body hydration.

This study was conducted as an attempt to elucidate the possible involvement of papillary HA in renal water handling. For this purpose, we measured the HA contents in the cortex, outer medulla and inner medulla (papilla) of kidneys from normal rats under different physiological situations of renal water handling (euvolemia, water diuresis and anti-diuresis) and from animals with a hereditary difference in renal water handling (desert rodents=gerbils; Brattleboro rats=Diabetes Insipidus). Furthermore, the effect of the vasopressin (ADH) V₂-receptor agonist desmopressin was tested on renal HA levels in normal rats, subjected to water loading. The results show that in all normal rats investigated, the papillary HA content was approximately 100-200 times higher than that of the cortex, while the corresponding values for the outer medulla was 10-30 times higher. In gerbils and Brattleboro rats, the papillary content was about 50 times higher than in the cortex. The amounts of HA in the papilla and outer medulla were considerably lower in gerbils than in normal rats. The amount of HA in the outer medulla of rats with Diabetes Insipidus (Brattleboro rats) was higher than that of normal rats, while the papillary content was similar. The cortical HA content was low and similar in all groups of animals, except in the Brattleboro rat which had elevated levels.

Regarding the histochemical distribution of HA, positive staining was seen in the interstitium of the papilla, but there was no staining in that of the cortex. More staining was found in the papilla of water-diuretic animals than in that of euvoletic animals. Furthermore, considerably more HA staining was evident in the outer medulla and papilla of normal rats than those of gerbils.

There was a direct correlation between papillary HA and water content, while an inverse relationship was seen between papillary HA content and urine osmolality. In the euvoletic group (control group), the content of HA in the papilla was 421±29 µg/g dry weight (dwt) and the papillary water content was 83.09±0.43%, see Fig.1. In animals subjected to water diuresis, papillary HA was 48% higher and its water content was increased by 4%, as compared to the control animals. The addition of a short-term treatment with desmopressin under water diuresis did not significantly alter
the papillary HA and water contents, as compared to animals not treated with desmopressin. 24h of anti-diuresis decreased the papillary HA by 17% and the water content by 2% (Fig. 1). The papillary HA and water contents of gerbils were lower than those in control rats. In the Brattleboro rats with Diabetes Insipidus, both the outer medullary and the cortical contents of HA were higher as compared to those of normal rats, while the papillary content was similar.

Figure 1. Papillary HA and water content. * denotes $p<0.05$ as compared to euvoletic control rats. GER, gerbils; AD, anti-diuresis; EUV, euvoletic control; WD, water diuresis and WD+D, water diuresis and 20 min desmopressin.

In conclusion, the amount of interstitial HA in the renal papilla is related to the water balance. We suggest that during induced water diuresis, increased interstitial HA might antagonise water reabsorption, while decreased interstitial HA will facilitate water reabsorption, which may involve changes in the interstitial hydrostatic pressure. The findings in gerbils and Brattleboro rats support this notion. The potential of interstitial HA as a regulator of renal water excretion points to a need for further studies on the mechanisms regulating the synthesis, degradation and elimination of renal papillary HA.
II. Renomedullary interstitial cells in culture; the osmolality and oxygen tension influence the extracellular amounts of hyaluronan and cellular expression of CD44.

In this investigation, we sought to determine the possible mechanisms underlying the changes observed in Study I. For this reason, we performed studies on cultured rat RMICs, with the aim of answering the following questions: a) Is the HA content expressed by RMICs dependent on local environmental factors such as the osmolality and oxygen tension, considering that the osmolality and oxygen tension of the renal papilla vary with the medullary blood flow and water balance? b) Do RMICs express the hyaluronan-binding protein CD44? c) If so, is CD44 influenced by changes in osmolality and oxygen tension?

Under normoxic conditions (20% O₂), the HA content of supernatants of sub-confluent cells under isotonic conditions (330 mOsm/kg H₂O) was 120±37 pg/10⁴ cells/24 h. This increased under hypo-osmotic and decreased under hyper-osmotic conditions (Fig. 2). Confluent RMICs also responded to hyper-osmotic challenges by a decrease in HA content. However, both the absolute values and the percentage decreases were lower as compared to sub-confluent cells.

![Figure 2](image)

**Figure 2.** HA content of supernatants of subconfluent and confluent RMICs following a manipulation of osmolality. *p<0.05 vs isotonic conditions (330 mOsm/kg H₂O), #p<0.05 vs subconfluent cells.
Under isotonic conditions, the HA content of supernatants from sub-confluent cells under normoxic conditions (20% O₂) was 107±12 pg/10⁴ cells 24 h. A reduction in the oxygen tension to 5% and 1%, respectively, resulted in a reduction in the HA content. The HA levels in the supernatants from the confluent cultures were lower than those from the sub-confluent ones, as expressed per cell.

Both sub-confluent and confluent RMICs showed positive immunostaining for CD44. An apparent increase in antibody binding was observed with gradual increases in osmolality. Flow cytometric analysis confirmed that successive increases in osmolality resulted in increases in the mean fluorescence intensity, representing increases in the number of CD44 receptors (Fig. 3).

![Figure 3. CD44 expression on RMICs grown in culture media with different osmolality.](image)

* denotes p<0.05 as compared to control (isotonic media, 330 mOsm/kg H₂O).

*In conclusion*, cultured rat RMICs produce HA; the extracellular content of HA is regulated by changes in local environmental conditions such as osmolality and oxygen tension. Furthermore, the expression of an HA-binding protein, CD44, is influenced by the same environmental factors. These findings support our previous *in vivo* observations of a decreased papillary HA content during antidiuresis and an increased content during water diuresis.
The results suggest that RMICs play an important role in renal water handling by regulating the amount of HA in the papillary interstitium and thereby determining the physico-chemical characteristics influencing osmotic activity and water transport.

**III. Renomedullary and intestinal hyaluronan content during body water excess.**

**A comparative study in rats and gerbils**

Our previous *in vivo* and *in vitro* studies in rats have suggested a role for renomedullary HA in water handling. In the gerbil, we found low levels of papillary HA compared to that of rats in normal physiological conditions. The gerbil is known for its unique ability to conserve water by producing concentrated urine. In the present study, renal papillary HA was compared between groups of anesthetized gerbils and rats before and after up to 6h of induced water diuresis (i.v. infusion of hypotonic saline).

The heterogenous intrarenal distribution of HA was confirmed in both species with hundred-fold higher amounts of HA found in the renal papilla as compared to the cortex per tissue weight. Papillary HA in gerbils was only 37% of that in the rat. In line with our previous investigations, water diuresis in rats increased the papillary HA content. The elevation was maximal (+27%) after 2h of water diuresis and then declined towards control levels at 4 and 6h of diuresis (+3%). In contrast, the gerbil responded with a decreased papillary HA content during water diuresis. The depression was maximal after 2h of diuresis (-49%) and was still 41% below the control values after 6h of diuresis (Fig. 4).

The urine flow rate increased rapidly in the rat and its maximum occurred at the HA peak, i.e. after 2h of water diuresis (twenty-one times above the control group) while in the gerbil, the urine flow rate increased slowly and slightly and was only six times higher than the control values after 6h of diuresis. The HA content along the intestine was similar in the two species and it was lowest in the duodenum and jejunum and highest in the distal colon (Fig. 5). The HA content along the intestine remained unchanged during water diuresis.
Figure 4. Percentage change in renal papillary HA content during control conditions (Time 0, C) and during up to 6h of induced water diuresis in gerbils (filled bars) and in normal rats (hatched bars). *denotes p<0.05 vs control for the respective species.

Figure 5. HA content along the intestines of gerbils (filled bars) and rats (hatched bars). Abbreviations: DUO, duodenum; JEJ, jejunum; ILE, ileum; PRC, proximal part of colon; DIC, distal part of colon.

In conclusion, in the rat, the elevation of papillary interstitial HA during acute water loading would counteract water reabsorption by changing the physico-chemical
characteristics of the interstitial matrix favoring water diuresis. In combination with reduced levels of ADH-regulated aquaporins, this would result in a rapid and major diuretic response. The gerbil has a diametrically different regulation of papillary HA turnover during water loading. The decreased papillary HA level during water loading and the slow and small diuretic response may represent a genetic difference in adaptation in this strain to ensure the ability to conserve water in an arid environment.

IV. Renal cortical hyaluronan during ischemia-reperfusion injury.
Accumulation of HA occurs in the renal cortex in several pathological conditions and is paralleled by the development of interstitial oedema. The aim of the present study was to investigate if renal cortical HA accumulation correlates with renal function after renal ischemia-reperfusion (IR) injury. Furthermore, the aim was to investigate whether treatment with the HA-degrading enzyme hyaluronidase will improve renal function after an IR injury. After 20, 30 or 45 min of left uni-lateral isothermic renal ischemia and 72h of reperfusion with daily injections of hyaluronidase or vehicle or continuous infusion of hyaluronidase or vehicle for one hour on day 3, renal function was studied and cortical HA content were measured. The vehicle treated IR injured kidneys showed poor concentrating ability and reduced GFR and RBF as compared to the contra-lateral control kidney (Fig. 6).

Figure 6. GFR in the left IR injured kidneys after different time periods of ischemia.
* denotes p<0.05 as compared to 0 min=control.
The amount of cortical HA was correlated to the time period of ischemia: the longer the ischemic periods, the larger the accumulation of cortical HA (Fig. 7). The IR injured kidneys did, on average, have ten-fold higher amounts of cortical HA than the control kidney and elevated water content. GFR was inversely correlated to the amount of HA.

![Graph showing cortical HA in the left IR kidneys after different time periods of ischemia.](image)

*Figure 7. Cortical HA in the left IR kidneys after different time periods of ischemia.*

* denotes p<0.05 as compared to 0 min=control.

Hyaluronidase treatment did not significantly alter the total cortical HA and water content or improve the kidney function in the IR injured kidney. The HA content of the outer medulla of the contra-lateral kidney did, however, slightly decrease during hyaluronidase treatment, thereby indicating an active enzyme.

**In conclusion,** a renal IR injury depresses parameters of renal function, which coincides with a severely elevated cortical HA content and an interstitial oedema. The severity of cortical HA accumulation and depression of GFR is correlated to the time periods of ischemia. The water binding properties of HA and the resistance to flow suggest a causal relationship to the depression in GFR. Hyaluronidase injections failed to reduce cortical HA levels and significantly improve renal function after IR injury. However, further studies using alternative modes of hyaluronidase administration are needed to fully elucidate the role of hyaluronidase treatment in this pathological setting.
DISCUSSION

The kidneys are the major organs responsible for maintaining a relatively constant volume and a stable composition of the body fluids, which is essential for homeostasis. By controlling the rate at which the kidneys excretes water and electrolytes, a fine balance between input and output can be achieved. Several mechanisms cooperate to achieve this goal. A structural component, which has only been slightly considered in this respect, is the interstitial matrix component hyaluronan (HA). HA is a large, linear, negatively charged glycosaminoglycan found in the interstitium of the kidney (Pitcock et al 1988, Hällgren et al 1990a) with a heterogenous distribution. Since it was found in the highest concentration in the inner parts of the kidney, our hypothesis was that it should be of importance for water handling. This was also supported by early findings in changes in the amount of glycosaminoglycan of the papilla and in the HA-degrading enzyme hyaluronidase excretion with changes in water balance (Ginetsinsky 1958, Rowen & Law 1981). The overall conclusions of the present investigation are that medullary HA and renomedullary interstitial cells, RMICs, play an important role in renal water handling. Furthermore, the intrarenal distribution of HA seems to be of importance for the proper function of the kidney. Changes in the distribution of HA occur during renal ischemia-reperfusion (IR) injury, which coincides with a severe depression of the renal function.

The involvement of HA in the kidney function was implicated already in 1958, when Ginetzinsky published a paper on the changes of hyaluronidase in urine in relation to the water balance of the organism. The amount of hyaluronidase increased with anti-diuresis and dropped to zero after water loading. Rowen and Law then confirmed this finding in other studies in the late 1970’s and early 1980’s, where they suggested a functional relationship between the degradation of medullary mucopolysaccharides by hyaluronidase, and the concentrating ability of the kidney.

Our first in vivo study, Study I, suggested that HA plays an important role in the renal water balance. This conclusion is based on the findings of an increased papillary HA content during i.v. water loading and a decreased HA content during dehydration. The possible underlying mechanism of these findings was the next question we attempted to answer. To mimic the milieu of the renal medulla in vivo under conditions of water diuresis and anti-diuresis, we examined cultured RMICs,
following changes in osmolality and oxygen tension and measured the extracellular HA content and the cellular expression of the HA-binding protein, CD44. The importance of renal HA was then investigated in a species adapted to an arid environment, the gerbil, which was compared with the normal rat. We studied the papillary interstitial HA when these species were subjected to body water excess (water loading) creating water diuresis. The results showed that the response of the rat and that of the gerbil upon water loading are diametrically different, probably reflecting an adaptational difference to the arid environment of the gerbil. The last issue raised was the one concerning the change in the distribution of HA under pathological conditions, namely after renal IR injury. We confirmed the finding of others regarding the accumulation of HA in the interstitium of the cortex after renal IR injury (Johnsson et al 1996, Lewington et al 2000) and, furthermore, correlated it to a depressed function of the kidney. Treatment with the HA degrading enzyme, hyaluronidase, was not successful in this setting.

**HA and fluid handling**

In *Study I*, we investigated the HA content in the kidney in different states of body hydration. Both rats and gerbils were used and we also studied Brattleboro rats, known for their defect in water handling. The results confirmed earlier findings with HA normally present almost exclusively in the renal medullary interstitium and not in the cortex. Furthermore, the HA content of the papilla is increased during acutely induced water diuresis (+48%) and modestly, but significantly, decreased during dehydration (-17%). In gerbils, papillary and outer medullary HA contents were only 25% and 13%, respectively, of those in normal rats, while the cortical content was similar. In Brattleboro rats, the outer medullary HA content was significantly higher (+285%) than in the normal rat, while the papillary content was similar.

A positive correlation between the HA content of the renal papilla and the urine flow rate and an inverse relationship to urine osmolality were also evident. These results suggest that HA has a role in renal water handling, probably by changing the interstitial matrix of the medulla, which may influence the water transport properties of the medullary interstitium and the interstitial hydrostatic pressure. The net charge of HA has been estimated at 5-10 mEq/l (Öjteg et al 1988), which at physical ionic
strength will result in an electoosmotic pressure of 1-3 mmHg. In a free space, this pressure will result in absorption of fluid by the HA gel with subsequent expansion and thereby also disintegration, a process which obviously will be accompanied by a parallel reduction of the osmotic pressure. However, if HA is enclosed by the vascular and tubular structures of the kidney, the fluid absorption will instead increase the hydrostatic pressure of the gel such that it reaches the same 1-3 mmHg. This means that the spatial distribution of the above mentioned structures might in fact be determined by the extracellular HA gel.

The amounts of extractable HA in renal papilla were impressive (on average 421 µg/g dry weight tissue) and are about 100 times larger than those in the renal cortex and 10-30 times larger than those in the outer medulla. The amounts of HA in the renal papilla are also larger than the amounts extractable from various other organs, e.g. the heart, lung and small bowel (Waldenström et al 1991, Nettelbladt et al 1989, Wallander et al 1993). The mechanisms underlying this heterogeneous distribution of HA in the kidney are not fully understood. Theoretically, the large amounts in the papilla could be due either to an enhanced local production of HA, reduced elimination or degradation of HA or a combination of the two. One hypothesis regarding this distribution was examined in Study II, where we investigated the RMIC and how the osmolality and oxygen tension affect their expression of HA and CD44. The idea was that low oxygen tension could be a trigger for increased HA expression by RMICs, which may be amplified during an ischemic insult. This was based on two findings: first, the oxygen tension in the renal papilla is lower than that in the cortex, because of the counter-current arrangement of vasa recta shunting oxygen from descending to ascending vessels, and second, that during renal IR injury, the HA content of the renal cortex increases dramatically. In Study II, we found that the HA content decreases upon exposure to reduced oxygen tensions, which is probably a consequence of a reduction of HA synthesis, which is, in itself oxygen-dependent. However, in the pathological situation of IR injury, it is likely that the release of, for example cytokines and other growth factors from infiltrating cells of the immune system or other cells influence the HA turnover, which has been seen in other systems (Spicer et al 1996, Heldin et al 1989, Jones et al 2001).
One of the enigmatic areas in HA metabolism is the lymphatic system. The major proportion of the circulating HA is present in the lymphatic system (Fraser & Laurent 1989). The major part of the tissue HA is normally supposed to leave the interstitial compartment by lymph drainage and then be degraded by the regional lymph nodes before reaching the general circulation and being absorbed by the liver (Eriksson et al 1983, Fraser et al 1988). The almost complete lack of lymph drainage from the papilla as opposed to that of the renal cortex might therefore contribute to the high papillary HA content and the small amounts found in the cortex. Enzymatic local degradation of HA may also be of importance for the HA clearance. The enzyme hyaluronidase is known to occur in the rat kidney (Bollet et al 1963, Sun et al 1998). It has been shown that the hyaluronidase activity is low in the outer parts but increases significantly towards the tip of the papilla (Goryunova et al 1975) suggesting that the local turnover of HA is rapid here.

It is conceivable that RMICs are the main HA producers in the papilla. This fibroblast-like cell produces HA in culture. In Study II, we found that RMICs in culture expressed less HA under hyperosmotic conditions thereby confirming our previous results (Hansell et al 1999). The RMICs produce more HA under hypoposmotic conditions. Further on, we investigated the HA binding protein, CD44. Binding of HA to CD44 may be followed by receptor-ligand internalisation and degradation of HA by acid hydrolysis in lysosomes, as has been shown for several cell-types. Thus, decreased expression of CD44 associated with decreased osmolality during water diuresis may result in a decrease in uptake and degradation of HA, leading to an increased HA content in the interstitium (or in the culture medium as shown in Study II). To confirm our own findings and to elucidate if the CD44-receptors found on RMICs were active, we repeated this experiment by pre-treating the RMICs with hyaluronidase before measuring the CD44 expression and thus observed the same pattern, i.e. an increasing amount of receptors with increasing osmolality and vice versa. The CD44-receptors present on RMICs were capable of binding FITC-labelled HA in the same manner (unpublished data).

The findings regarding RMICs and CD44 would fit well with the finding in Study I that dehydration (high urine osmolality) caused a decrease in the papillary HA
content and that water loading (low urine osmolality) caused an increase in the papillary HA content. Physico-chemical studies on HA in vitro have shown HA can become entangled, forming a network occupying the solvent space and excluding large molecules. This network-like matrix may affect osmotic activity of, and fluid transport in, the interstitium (Comper & Laurent 1978, Scott 1989, Laurent & Fraser 1992).

The accumulation of HA in the interstitium causes an interstitial oedema, which can be called a “functional oedema” in this physiological setting since the structures of importance for water reabsorption are being separated. The HA gel keeps the structures apart from each other, actually determining the spatial distribution of the interstitium. With increasing distance, the diffusion rate is decreased accordingly. The presence of HA would also resist hydraulic flow, due to the “gelling” properties of the molecule. Another theory regarding the role of HA in the interstitium is that HA is of importance for maintaining the concentration gradient of the medulla. This gradient is due to the progressive accumulation of sodium, chloride and urea towards the tip of the papilla. The polyanionic HA causes a gradient of negative charges, which can sequester ions and contribute to the high osmolality.

During water loading, the relative water reabsorption needs to be decreased in the medullary collecting system. Therefore, we suggest that increased interstitial HA in concert with decreased numbers of ADH-regulated aquaporins antagonise water reabsorption. The RMICs produce more HA and express less CD44 thereby creating an increased amount of HA in the interstitium. The antagonism of water reabsorption by interstitial HA may involve changes in the interstitial hydrostatic pressure as well as a resistance to flow. The idea that HA decreases water transport is in line with the reports by Wang et al (1998 and 1999) that an intra-peritoneal injection of HA decreased peritoneal fluid absorption. Furthermore, it has been demonstrated that HA diminishes the water permeability of the interstitium (Lai-Fook & Brown 1991, Zawieja et al 1992).

In vitro experiments show that an HA-gel reduces the diffusion of water molecules (Cowman et al 1998). Other in vitro models also find that the properties of the HA gel is affected by the osmolality, or ionic strength, of the surroundings. The presence of, for example NaCl, in the gel causes the gel to behave differently, it
increases self-diffusion as well as tracer-diffusion with increasing concentrations (Gribbon et al 1999 and 2000). These findings might have consequences for our model, for this suggests that there is not a linear relationship between the amount, or concentration, of HA and the properties of the gel, but that other factors must also be considered. In our in vivo model, we can assume that we have changes in electrolytes/osmolites and can therefore hypothesize that the properties of the gel-like matrix change accordingly.

Our finding in Study I and III that there was a significant increase in the papillary interstitial HA only 2 hours after the start of water loading may seem rather fast, at least if the HA increase is due to enhanced local synthesis. However, our histochemical analysis gave positive support to the quantitative data. In pathological conditions enhanced HA synthesis becomes apparent within 1-2 days. Thus, we speculated that the HA accumulation in the renal papilla was more likely to be due to influence on the normal route of elimination/degradation of papillary HA, e.g. down-regulation or blockage of enzymatic digestion of HA. It has, however, recently been shown that the HA synthases, HAS, can change their expression in 6 hours in vitro (Jacobson et al 2000). We can therefore not rule out that a changed synthesis per se could be involved as well as a change in the degradation of HA. As for now, we have no other data on the behavior of the HAS (HAS1-3), which have all been demonstrated in the kidney (Spicer & MacDonald 1998, Feusi et al 1999), during water loading.

If HA does play a physiological role in renal water handling, it is conceivable that the papillary HA content decreases during dehydration in order to facilitate water reabsorption. Our experimental studies support this hypothesis. During dehydration of normal rats for 24 hours their urine osmolality increased by 27% on average and their papillary HA decreased by 17% on average. RMICs grown in a culture with media osmolalities higher than 330 mOsm/kg H2O, i.e. 630, 930 and 1,230 mOsm/kg H2O produced less HA and expressed more CD44, probably leading to an increased uptake and degradation of HA. ADH is certainly involved in enhanced water reabsorption during dehydration and renal hyaluronidase has been proposed as a mechanism of action of this hormone (Ivanova & Meldini 1999). In this context, it is worth noting that short-term (20 min) administration of the ADH V2-receptor agonist desmopressin
had no influence on the papillary HA content. Further studies using prolonged exposure to ADH should be performed to conclude how this hormone is involved in the HA turnover.

Further support for a physiological role of HA in water handling was provided by the finding that gerbils had considerably smaller amounts of HA, both in the papilla and in the outer medulla compared with normal rats. This observation suggests that in the gerbil, the ability to reabsorb water is at least partly increased as a result of an altered extracellular matrix. A tubular system with large amounts of ADH-regulated aquaporins situated in such an environment should allow excessive water reabsorption. We investigated the consequences of up to 6h of water loading on papillary HA content in rats and gerbils. It is clear from the results of Study III that the gerbil has a completely different regulation of papillary HA as compared to the rat: the elevation in papillary HA which occurs in the rat during water loading is diametrically changed into a decrease. In the rat, 2h of water loading leads to an increase in HA and the response then normalizes. In the gerbil, water loading leads to a decrease in papillary HA and the reduction persisted during the entire 6h of water loading.

It is interesting to note that in the rat, the HA peak occurs simultaneously with the peak urine flow rate at 2h and that the latter levels out at 4 to 6h of water loading, at about ten times above the control levels. It could be speculated that papillary HA in the rat is primarily important for the rapid and acute excretion of water during excessive intake. For the gerbil, it is clear that the diametrically different response, i.e. the depression in papillary HA, does persist for at least the 6h duration of the induced water diuresis and that the diuretic response is slow and relatively small during this period. It could thus be suggested that papillary HA in the gerbil is not only important in the acute phase of water loading as is the case in the rat, but seems to be important for a longer period of water intake. In a study on the activity of the hypothalamus-neurohypophysis during rehydration (Edwards 1984), provided further evidence of this. He showed that water deprivation for 3 or 5 days resulted in the same degree of ADH depletion, but that the ADH stores were replenished more quickly in the gerbil. Gerbils also returned more quickly to their normal body weight (also shown by others, McManus 1972). Gerbils produced small volumes of more concentrated urine even
24h after free access to water, while the rat produced urine of similar volume and concentration as the control animals. This might explain the differences in body weight but also suggests that gerbils have higher concentrations of ADH, even after water deprivation (the rehydration phase) (Edwards 1984).

As to the mechanism underlying the reduction of papillary HA with water loading in gerbils, no in vitro studies have investigated the influence of osmolality on CD44 receptor regulation on RMICs in this strain as we have done in the rat. We can therefore not conclude if the CD44 regulation on RMICs is different in gerbils vs rats in response to changes in osmolality.

In Study III, we also investigated the presence of HA along the intestine under control conditions and after i.v. water loading both in gerbils and rats. There was no species difference, neither in the amount of HA nor in its distribution. The HA content in rats and gerbils was lowest in the duodenum and jejunum and was about four times higher in the distal colon (values similar to those found in the renal papilla). The kidney and the intestine are the two major organs involved in transport of fluids. It is interesting to note that high and low amounts of HA in different regions of both the kidney and the intestine correlate with regions where small and large fluid volume transports occur. In the kidney, the major fluid volume reabsorption occurs in the cortex where almost no HA is found. On the other hand, very high amounts of HA are detected in the papilla, where less fluid volumes are reabsorbed. It is, however, important to acknowledge that the regulation of the fluid balance occurs in the medulla as opposed to the cortex (Guyton & Hall 1996). The same line of reasoning applies to the intestines. The lower levels of HA are found in the duodenum and jejunum, where a large part of the fluid absorption takes place. The highest amounts of HA are found in the colon, which actually absorbs less fluid volumes but stands for the final regulation of fluid absorption.

I.v. water loading did not change the HA concentration along the intestine, which was not surprising. Giving similar amounts of fluid orally would probably be a better model to elucidate if a similar change in HA levels occurs in the intestines as in the kidney or if a different HA regulation is at hand. It is also important to remember that the major species difference in HA content observed in the papilla is not found in
the intestine, where quite similar levels are detected. This might be explained by the fact that the healthy intestine absorbs practically all the water ingested and that the main function of the gastrointestinal tract in water homeostasis is to deliver water to the systemic circulation (Turnheim 1984). Renal mechanisms, on the other hand, accomplish the preservation of the water equilibrium of the whole organism.

Gerbils and rats differ in several respects. Regarding the kidney, there are anatomical and physiological differences between the species as mentioned in the introduction. A comparison between rats and gerbils of their tolerance of 2% (0.34 M) NaCl in their drinking water shows that this concentration rapidly causes dehydration of the body fluids and a massive depletion of ADH in the rat, while the gerbil showed little or no alteration in body hydration (Donaldson & Edwards 1981). In an investigation of the distribution of water in the gerbil during water deprivation it was shown that the ability of the gerbil to maintain plasma volume was better than that of the rat (Edwards 1991). The maintenance of plasma volume at the expense of other fluid compartments can be seen as an adaptation in a hot environment, for it would allow the circulation to carry away metabolic heat to the surface, thereby preventing a rapid and major heat rise.

Study III gives us yet another piece of information to describe different mechanisms of the rat and the gerbil to attain fluid homeostasis, which are probably genetically related to the environmental living conditions of the respective species. The diametrical difference in response regarding papillary HA regulation and water diuresis might partly explain how the gerbil can retain so much fluid after up to 2h of water diuresis (it retains about 50% of the infused volume) while the rat is close to equilibrium (retains about 15%). The goal for the reverse response regarding papillary HA regulation may lie in the need for the gerbil to survive in a more arid environment than the rat.

The water-binding properties of HA are reflected in inflamed or infarcted tissues, since the degree of tissue oedema associated with these conditions is related to the degree of interstitial HA accumulation (Hällgren et al 1990a, Waldenström et al 1991, Johnsson et al 1996, Nettelbladt et al 1989). In the healthy rat, tissue hydration was highest in
the HA-rich papilla with its calculated HA concentration of 0.6 mg/ml in normal physiological conditions. In Study I, the water content of the papilla varied from 81% during anti-diuresis to 86% during water diuresis and similar values were found in Study III. The observed correlation between the papillary content of HA and the degree of papillary hydration suggests that the interstitial HA is of importance not only in inflammatory oedema but also in the physiological regulation of interstitial water.

In Study II, confluent RMICs, as opposed to sub-confluent cells, responded with a smaller reduction in the HA content of their supernatant when exposed to a hyperosmolar milieu, and their basal HA content was significantly lower. Furthermore, confluent cells did not respond with an altered HA content, following reductions in oxygen availability. The mechanism underlying this phenomenon is not fully understood, but it may be attributable to the fact that HA synthesis mainly takes place in periods of growth and differentiation (Laurent & Fraser, 1986). Jacobson et al (2000) showed in human mesothelial cells that the amount of HA expressed by these cells as well as the expression of HAS2 were higher in sub-confluent than in confluent cultures. This notion is further supported by the finding of the preferential expression of CD44 on actively proliferating cells (Culty et al 1990, Alho & Underhill 1989). The phenomenon is often referred to as “contact inhibition”.

From Studies I, II and III, we conclude that the amount of interstitial HA in the renal papilla is related to the state of water balance. We suggest that during water loading increased interstitial HA could antagonise water reabsorption, while during dehydration decreased interstitial HA, will facilitate water reabsorption. This may involve changes in the interstitial hydrostatic pressure due to the unique water binding properties of HA, as well as the “functional oedema” which separate structures of importance for water reabsorption. The data from Study II on the RMICs and their expression of CD44 gives us a possible mechanism for the complex regulation of interstitial HA. The potential of HA as a regulator of renal water excretion points to a need for further studies of the mechanisms regulating the synthesis, degradation and elimination of renal papillary HA.
Redistribution of HA under pathological conditions

HA is also involved in several pathological conditions such as myocarditis (Waldenström et al 1993), alveolitis (Nettleblad et al 1989) and pancreatitis (Johnsson et al 2000), to mention a few. Accumulation of HA in rejecting organs (kidney - Hällgren et al 1990a, Wells et al 1990, heart – Hällgren et al 1990b, intestine - Wallander et al 1993, Johnsson et al 1993) is also known to occur.

The aim of the last investigation in this thesis, Study IV, was to investigate if a cortical accumulation of HA after renal IR injury alters the kidney function and if treatment with the HA degrading enzyme hyaluronidase during renal IR injury improves the kidney function. The results show that renal IR injury depresses parameters of renal function which coincides with a severely elevated cortical HA content, on average ten times higher than under normal conditions, and an interstitial oedema. The severity of cortical HA accumulation and reduction of GFR is correlated to the time period of ischemia. The cortical HA content is inversely correlated to GFR. Although this study does not provide proof of a causal relationship between the accumulation of cortical HA and the depression of renal function after IR injury, the physical and pro-inflammatory characteristics of HA may very well influence such a depression. The modulation of the interstitial HA concentration might be viewed as a mechanism for the organism to create an environment where tissue repair can take place (Gerdin & Hällgren 1997).

HAS1-3 has been demonstrated in the kidney. As to the cellular identity of the HA synthesis, RMICs produce HA and regulate the HA turnover in the papilla depending on growth media osmolality as shown in Study II. So far, no studies have been published to show if cortical fibroblasts also produce HA. During normal physiological conditions, the renal cortex is virtually void of HA. It is important to consider the occurrence of lymph vessels in the renal cortex, as opposed to that of the papilla, which may continuously drain a possible cortical fibroblast HA production. The possibility of the cortical fibroblast to respond upon stimulation has not been investigated. We do, however, have some preliminary data on the expression of HAS2 and HAS3 in ischemic kidneys, showing a strongly increased cortical HAS2 mRNA expression after IR injury. We did not see any alterations in the HAS3 expression (by means of RT-PCR, unpublished data). As to the mechanism of the cortical elevation of
HA during IR injury it is known that cytokines, which are released after renal injury (Jones et al 2001) can stimulate fibroblasts as well as tubular epithelial cells to increase HA expression (Heldin et al 1989, Hamerman et al 1984, Feusi et al 1999). Previously, we have demonstrated that it is not the hypoxia per se which triggers RMICs to increase HA expression which could also be true for cortical fibroblasts in this in vivo model.

Using the present mode of administration, hyaluronidase treatment failed to reduce renal cortical HA levels or significantly improve renal function in the IR injured kidneys. A defective enzyme and an impaired access of the enzyme to the damaged renal cortex might be part of the explanation. The former reason can easily be ruled out since we find that the outer medullary HA content in the contra-lateral control kidneys is reduced in response to hyaluronidase treatment, which would not occur if the enzyme were defect. In the present study, it is clear that the perfusion of the damaged kidney is reduced, which suggests that reduced amounts of hyaluronidase will reach the damaged kidneys when compared to the contra-lateral control kidney with intact perfusion. The use of gas-anesthesia (Isoflurane) might also explain some of our problems. It has been speculated that Isoflurane reduces renal blood flow, a fact that may contribute to the perfusion problem as well as being part of the explanation to the severity of the ischemic damage. Altogether, this may explain the negative results of the hyaluronidase treatment. To avoid one of these problems in future studies, the injection of a high concentration of hyaluronidase directly into the renal artery of the damaged kidney could be tested.

It could not be proven that the molecule has penetrated the cortical vessels of the damaged kidneys to reach the interstitial space where the accumulated HA resides. However, the size of this hyaluronidase is approximately 55 kDa and the fenestrated endothelium of the peritubular capillaries should allow passage of the enzyme, especially in the damaged kidney where the permeability should be enhanced compared to that of normal capillaries. It was part of our first hypothesis regarding the hyaluronidase treatment that it should be selectively taken up by areas of damaged capillary membrane, according to the permeability theory of IR injury (Wolgast et al 1982), which states that capillary permeability is increased so that not only water but also large molecules like proteins (and hyaluronidase) can leak into the interstitium.
The damage of the capillary membrane is attributed to the effects, indirect or direct, of reactive oxygen species, generated during the early recirculation (Wolgast et al 1986, Weinberg 1991). In addition, it has previously been shown that the uptake of hyaluronidase is facilitated in damaged tissue (Johnsson et al 2000).

One last hypothesis regarding the failure to reduce HA with hyaluronidase treatment might be that hyaluronidase stimulates the synthesis of HA, which has been shown in vitro (Philipson et al 1985, Lüke & Phrem 1999). This feedback mechanism could cause the HA content of the IR injured kidneys to be elevated, due to a hyaluronidase-stimulated synthesis. These studies have shown both an increase in the rate of elongation of the HA-chain and in the release-rate of the chain from the enzyme complex. However, this would probably be more of a problem when giving daily infusions of hyaluronidase rather than when using one single continuous infusion at the end of the experimental period.

Our negative results regarding hyaluronidase treatment is at variance with a previous study showing reduced renal HA levels from hyaluronidase treatment in IR injured kidneys (Johnsson et al 1996). This investigation did, however, not study renal function after treatment and did not investigate which intrarenal section was responsible for the reduction in total HA content. Furthermore, it is clear that the ischemic damage was more severe in the present study than in the study by Johnsson et al (1996), which may partly explain the differing results.

In the cortical areas staining positive for HA at the histochemical analysis, infiltrating immune cells were also found. The reason for this co-existence might be twofold: 1) HA could promote the homing of inflammatory cells to the injured tissue (Mikecz et al 1995) and 2) the inflammatory cells release factors (e.g. cytokines and other growth factors) inducing an HA synthesis via stimulation of the hyaluronan synthases (Hamerman et al 1984, Heldin et al 1989). HA fragments have been shown to stimulate chemokine gene expression in macrophages (McKee et al 1996 and 1997), which could also contribute to co-existence and the inflammatory response. Furthermore, the expression of CD44 is up-regulated in kidneys subjected to ischemia. In a recent study by Lewington et al (2000), no CD44 expression was found in the cortex of non-ischemic kidneys but one day after ischemic injury, mRNA for CD44
was evident. It primarily occurred in the proximal tubules undergoing repair and was found both in the basal and lateral membranes. In our model, there was also a strong up-regulation of the CD44-expression in the ischemically damaged kidney, which displayed a positive staining of both proximal and distal tubuli. Several other pathological models, e.g. models of tubulointerstitial nephritis and glomerulonephritis, also show increased cortical tubular CD44 expression (Sibalic et al 1997, Jun et al 1997). In these models, there was a correlation between the HA staining and the CD44 expression. The reason for this distribution and correlation is not clear but might play a role in HA removal and metabolism and in the regeneration of the tissue.

In conclusion, renal IR injury depresses parameters of renal function, which coincides with a severely elevated cortical HA content and an interstitial oedema. Further studies are required to claim a causal relationship between renal dysfunction and cortical accumulation of HA.
CONCLUSIONS

This thesis has shown that HA and RMICs are important for renal water handling and that the osmolality is important in regulating production and elimination of HA from the renal tissues. Ischemic injury causes cortical accumulation of HA, which coincides with a severe depression of functional parameters. In summary, we can conclude that:

- the amount of interstitial HA in the renal papilla is related to the state of water balance of the organism. During i.v water loading in rats the HA content is increased and a decreased papillary HA content is observed after dehydration. The elevation of papillary interstitial HA observed during acute water loading in the rat in combination with reduced levels of ADH-regulated aquaporins would result in a rapid and major diuretic response.

- the gerbil, as compared to the rat, has a low papillary HA content and a diametrically different regulation of papillary HA turnover during water loading. The decreased papillary HA level in gerbils during water loading and the slow and small diuretic response may represent a genetic difference in the adaptation in this strain to ensure the ability to conserve water in an arid environment.

- cultured rat RMICs produce HA. The HA turnover is regulated by local environmental conditions such as osmolality and oxygen tension. Furthermore, the expression of an HA-binding protein, CD44, is also influenced by the same environmental factors. The present results suggest that RMICs play an important role in renal water handling by regulating the amount of HA in the papillary interstitium partly via the CD44 receptor, which is known for its role in the uptake and degradation of HA. By changing the HA amount in the interstitium, the physico-chemical characteristics influencing water transports can be altered.
renal ischemia-reperfusion injury depresses parameters of renal function, which coincide with a severely elevated cortical HA content and an interstitial oedema. The severity of the cortical accumulation and depression of GFR is correlated to the time period of ischemia. The cortical HA is inversely correlated to the GFR. Hyaluronidase injections failed to reduce cortical HA levels and significantly improve the renal function after ischemia-reperfusion injury but further studies using different application protocols are needed to fully elucidate the role of hyaluronidase treatment in this pathological setting.
Kortfattad bakgrund

Njuren är det i särklass viktigaste organet för regleringen av organismens vatten- och elektrolytbalans. Vid vattenbrist ställer njuren om sig så att en koncentrerad urin kan bildas och vid ett högt vattenintag bildas en utspädd urin. Flera olika system samverkar för att uppnå detta resultat. ADH, antidiuretiskt hormon, är den enskilt viktigaste reglerande faktorn då det gäller att ta upp vatten från de distala delarna av nefronet, primärt njurens märg, som framförallt styr vattenbalansen.

Hyaluronan, HA, är en stor, negativt laddad, linjär polysackarid som består av upprepade disackaridenheter. HA kan bildas av flera olika celler, finns på många platser i kroppen och har många funktioner. En viktig egenskap hos HA är dess förmåga att vid en viss koncentration bilda ett speciellt nätverk där vatten kan immobiliseras och en gel bildas. HA finns normalt heterogent fördelat i njuren med höga halter i de inre delarna, märgen/papillen, och nästan inget alls i de yttre delarna, barken. Detta kan vara av betydelse för njurens förmåga att koncentrera urinen. HA tros utöva sin effekt på vattenreabsorptionen genom att fungera som en barriär eftersom vattenrörelser hämmas av dess närvaro. Man har också sett att vid vissa sjukdomstillstånd i njuren (ischemi-reperfusions skada, rejektion) störs fördelningen av HA och de normalt låga halterna i de yttre delarna blir kraftigt förhöjda. Detta tros påverka funktionen hos njuren negativt, bl.a. genom bildandet av ett interstitiellt ödem.

Behandling med hyaluronidas, HA-nedbrytande enzym, kan möjligtvis minska halterna och därmed förbättra funktionen. Detta skulle i så fall kunna bli en ny behandlingsform vid organskador där HA-halten är onormalt förhöjd. Våra och andras resultat inom området visar på en viktig roll för HA i njurens vattenhantering och också på det faktum att fördelningen är av största vikt: då den rubbas störs njurfunktionen.

Sammanfattning av de arbeten som ingår i avhandlingen

I. Hyaluronan content in the kidney in different states of body hydration.

Hansell P, Göransson V, Odlind C, Gerdin B and Hällgren R.

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Den första studien utformades för att undersöka den möjliga inblandningen av renalt interstitiellt hyaluronan (HA) i regleringen av vätskebalansen. HA och vatteninnehållet i njurens olika delar undersöcktes i sövda råttor med olika vattenbalans (dehydrerade, vattendiures, kontrollsituation) samt i ökenrättor och rättor med Diabetes Insipidus (avsaknad av antidiuretiskt hormon, ADH). Resultaten visade dels att HA är heterogent fördelat i njuren (mycket i märgen, lite i barken) samt att HA regleras till följd av organismens vätskebalans. Vid vattenbelastning ökar HA-innehållet i njurens innersta delar (märgen, papillen) samtidigt som njurens vattenutsöndring ökar kraftigt. Förändringen sker snabbt, redan efter två timmar har HA-innehållet ökat med nära 50%. Vid törst gäller det omvända, HA-mängden och vattenutsöndringen minskar men omställningen är långsammare. Vattenmängden är vidare direkt korrelerad till njurens HA-innehåll. Ökenrättorna, vilka har en unik förmåga att koncentrera urinen vid behov, har mycket lägre HA-innehåll i papillen jämfört med normala rättor. Diabetes Insipidus rättor, vilka saknar förmågan att koncentrera urinen, har förhöjda halter av HA i den yttre märgen. De två senare fynden talar återigen för en viktig korrelation mellan HA och vattenhantering.


II. Renomedullary interstitial cells in culture: the osmolality and oxygen tension influence the extracellular amounts of hyaluronan and cellular expression of CD44.

I studie II användes renomedullära interstitiella celler, RMIC, för att undersöka förhållandet mellan osmolalitet, syrgastension och mängden HA, vilken producerats av RMIC i mediet. RMIC är troligen de huvudsakliga HA-producenterna i njurens märg, dvs den njurstruktur där vi i studie I fann höga HA halter vilka ändrades beroende av vätskebalansstatus. Vi undersökte också förekomsten av CD44, ett HA-bindande protein (receptor), för att kunna ge indikationer på en möjlig mekanism till renal HA-reglering. CD44 anses nämligen vara viktig för intracellulärt upptag och nedbrytning av HA. Resultaten visade att celler som odlades i medium med hög osmolalitet (jämför dehydrering i studie I) producerade mindre HA än de som odlades i isotont medium och att de celler som odlades i ett medium med låg osmolalitet (jämför vattendiures i studie I) producerade mest HA. Vidare fann vi att med ökande osmolalitet så ökade antalet CD44 receptorer på cellernas yta och vid lägre osmolalitet så fanns färre receptorer. Slutligen undersökte vi huruvida syrgastensionen påverkade HA innehållet i mediet eftersom man vid vissa tillstånd med låg syrgastension (ex. ischemi/reperfusion) ser en ökad mängd HA i njurens bark, där det normalt ej skall återfinnas i nämnvärda mängder. Detta visade sig dock inte vara fallet i cellkulturer: med sjunkande syrgastension minskade HA mängden vilket sannolikt återspeglar den syrgasberoende HA-syntesen i RMIC.

Sammanfattningsvis kan man säga att dessa in vitro-resultat stödjer våra in vivo-resultat i studie I och vidare ger en möjlig mekanism till njurens HA-reglering vid olika tillstånd av vätskebalans. Vid ökat behov av vattenutsöndring (vattendiures/låg medium- osmolalitet) ökar mängden HA, vilket bl a kan orsakas av att CD44 uttrycket minskar på dessa celler. Det senare minskar intracellulärt upptag och degradering av HA. Det omvända gäller vid dehydrering, dvs minskat behov av vattenutsöndring. Resultaten stödjer teorin om en viktig roll för RMIC och HA i njurens vattenhantering.

III. Renomedullary and intestinal hyaluronan content during body water excess.
A comparative study in rats and gerbils.
Göransson V, Johnsson C, Nylander O and Hansell P.

Sammanfattningsvis kan sägas att dessa två arter har olika basala HA-halter i njurens märg samt att de reglerar sitt HA-innehåll diametralt olika. Detta skulle tyda på en HA-reglering anpassad till organismens genetiskt ställda behov av vattenhantering.
IV. Renal cortical hyaluronan during ischemia-reperfusion injury.
Göransson V, Johnsson C, Hällgren R and Hansell P.

Vid olika typer av skador på njuren (ischemi-reperfusions (IR)-skada, rejektion efter transplantation) ser man en ackumulering av HA i njurens bark. Denna HA-ansamling anses vara kopplad till det interstitiella ödem som utvecklas vid exempelvis IR-skada. Den fjärde studien syftade till att undersöka huruvida denna HA-ansamling korrelerar till njurens funktion och om behandling med hyaluronidas (HA-nedbrytande enzym) kan förbättra funktionen och därmed fungera som en ny terapiform. Hypotesen var att det i den skadade njuren skulle ske ett selektivt upptag av hyaluronidas eftersom det är känt att IR-skada leder till ökad kapillärpermeabilitet. Vid försöken gjordes vänstra njuren ischemisk genom att stoppa blodflödet till organet medan den högra fungerade som kontroll. Ett flertal ischemitider provades (20, 30, 45 och 60 min) med samma reperfusionstid (72 timmar) och även två olika behandlingsätt, dels tre på varandra följande dagliga injektioner av hyaluronidas dels kontinuerlig infusion av hyaluronidas under två timmar. Funktionsparametrar så som urinproduktion, urinkonzentrationsförmåga och glomerulusfiltration (GFR) mättes samt parallellt med detta, HA-innehållet och vattenhalten i njurens olika delar. Resultaten visade att HA-innehållet i den skadade njurens bark var flera gånger högre än i kontrollnjuren och att detta gick parallellt med en förhöjd vattenhalt som visade sig som ett interstitiellt ödem vilket verifierades med morfologiska studier. Den skadade njuren hade också sämre urinkonzentrationsförmåga och GFR. Behandling med hyaluronidas påverkade HA-innehållet i vissa grupper, dock ej alla. Njurfunktionen avseende GFR och koncentrationsförmåga tenderade att förbättras i vissa IR-skadade grupper, dock ej signifikant. Det är inte klargiltigt om tillförseln av hyaluronidas till de skadade njurarna var optimal med tanke på deras sänkta genomblödning och om storleken på det använda enzymet var optimal med avseende på kapillärpermeabilitet. Dock minskade HA-innehållet i vissa delar av de normala njurarna, vilket visar på att enzymet var aktivt.

Sammanfattningsvis kan man säga att distributionen av HA är av betydelse för njurens funktion. IR-skada leder till en kraftig ansamling av HA i njurens bark,
vilket påverkar funktionen negativt. Fler studier krävs för att utröna huruvida hyaluronidas behandling kan bli värdefull vid denna typ av organskada.
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