Characterization of CD44-mediated hyaluronan binding by renal tubular epithelial cells

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Abstract

Background. CD44 is the main receptor for the extracellular polysaccharide hyaluronan (HA). We have recently shown that CD44 is strongly induced on renal tubular epithelial cells (TEC) in autoimmune renal injury and that HA accumulates in the renal interstitium. The functional significance of enhanced tubular CD44 expression and its interaction with HA are not known. The purpose of the present study was to characterize renal tubular CD44 expression and CD44-mediated HA binding in vitro and to investigate the growth modulating effects in response to HA binding by TEC.

Methods. RT-PCR analysis, flow cytometry, confocal microscopy and Western blotting were used to examine cell surface and soluble CD44 expression by cultured TEC, using SV40-transformed mouse cortical tubular (MCT) cells. HA binding characteristics were examined by flow cytometry and effects of HA on TEC cell growth by [³H]thymidine incorporation.

Results. By RT-PCR analysis MCT cells expressed predominantly the standard form of CD44 mRNA, whereas the expression of variant forms was very weak. Confocal microscopy showed that CD44 was expressed basolaterally and apically on MCT cells with strong staining on microvilli. Shedding of CD44 from MCT cells could be induced with crosslinking of anti-CD44 mAbs or with PMA stimulation. MCT cells constitutively bound HA and this binding could be modulated with anti-CD44 mAbs. Soluble and plate-bound HA markedly inhibited MCT cell growth.

Conclusions. CD44 is a regulated HA receptor on MCT cells which can be shed into the cellular environment. Upon binding of HA, CD44 functions as a growth inhibitory cell surface protein in MCT cells. We speculate that the interaction of CD44 with HA may have important regulatory effects on cell proliferation in tubulointerstitial renal diseases.

Key words: CD44; cell growth; hyaluronan; tubular epithelial cells

Introduction

The leukocyte antigen CD44 is a widely distributed cell surface proteoglycan which functions as a receptor for the matrix constituent hyaluronan (HA) [1]. Through its expression on non-haematopoietic parenchymal cells CD44 is involved in various biological processes, including cell migration, metastasis and inflammation [2].

We have recently shown that CD44 is induced on proximal tubular epithelium in immune-mediated renal injury, including murine lupus nephritis (MRL-Fas model) [3] and tubulointerstitial disease (CBA/CaH model) [4]. We also found that HA markedly accumulates in the renal interstitium in MRL-Fas mice (unpublished observation) and CBA/CaH-kdkd mice [4], particularly in areas where tubular epithelial CD44 is upregulated. The functional significance of CD44 expression on TEC, its mechanism of interaction with HA and its role in immune-mediated tubular injury are presently not known. We have speculated, however, that tubular CD44 could interact with its ligand in vivo and modulate tubular cell function in response to this ligand interaction [3].

Since CD44 is expressed by cultured renal tubular epithelial cells (TEC) [3] we have used a clonal TEC line to characterize the pattern of CD44 expression and its interaction with HA in vitro and to gain insight into the functional significance of CD44 expression on TEC. Here we show that a defined TEC line expresses high levels of the standard but not the variant forms of CD44, both apically and basolaterally. Furthermore, shedding of CD44 from TEC can be induced. CD44 binds HA constitutively and the binding capacity for HA can be modulated with mAbs targeting CD44. We also demonstrate that HA inhibits the growth of TEC through its ligand CD44. Thus, upon interaction with HA renal tubular CD44 functions as a growth inhibiting molecule. We speculate that the interaction of CD44 with HA could...
play a role in the regeneration and differentiation of injured TEC in vivo in various renal diseases with a tubulointerstitial component.

Materials and methods

Reagents

Tissue culture reagents were obtained from Life Technologies (Gaithersburg, MD) and chemicals from Sigma (St Louis, MO). Dr J. Lesley (San Diego, CA) provided us with the IRAWB14.4 hybridoma [5], producing an activating anti-CD44 monoclonal antibody (mAb) and with FITC-labelled HA. Purified sodium hyaluronate with a molecular weight of approx. 4–6 × 10^6 was obtained from Fluka (Buchs, Switzerland). Table 1 lists the target antigen, properties and source of the mAbs used in this study. The hybridomas for the mAbs IM7.8.1 [6], KM114 [7] and KMS81 [7] were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Hybridoma culture supernatants were purified using protein G-Sepharose CL-6B columns. KM114 was biotinylated according to a standard protocol.

Cell lines and cell cultures

An SV40-transformed mouse cortical tubular (MCT) cell line was used to study the expression and functional role of CD44 [8]. MCT cells were grown in tissue culture dishes in DMEM media supplemented with 10% FBS, 10 mM HEPES, 100 U/mL penicillin and 100 μg/mL streptomycin.

RNA extraction, RT-PCR and Southern blotting for CD44

Total RNA from cultured MCT cells was extracted as described [9]. Total RNA from murine kidney was isolated using lysis in guanidinium isothiocyanate and ultracentrifugation at 35000 r.p.m. through a CsCl gradient overnight [10]. The RNA was analysed for CD44 expression by RT-PCR as described [3] using a kit (Perkin-Elmer, Branchburg, NJ). For optimal detection of CD44 variant forms we used primers located in the CD44 sequence directly flanking the variant exon insertion site [11]. The upstream primer 5'-ACC CCA GAA GGC TAC ATT TTG C-3' and the downstream primer 5'-CTC ATA GGA CCA GAA GTT GTG G-3' were used to detect the presence of such variant forms. RT-PCR products were then resolved on 2% agarose gels.

To ensure specificity of the amplified fragments we performed Southern blot analysis and hybridization with a cDNA probe encoding for CD44 as described [3]. Blots were washed under stringent conditions (final wash in 0.2 × SSC, 1% SDS, 60 °C). Following hybridization the blots were exposed to Kodak X-OMAT AR film.

Flow cytometry

Flow cytometry was performed as described [3]. Briefly, cultured MCT cells were grown to confluence and were then detached with 5 mM EDTA in HBSS. Cells were washed once with Ca2+- and Mg2+-free HBSS and were resuspended in PBS containing 5% FCS. Cells were then incubated with the primary mAb for 1 h at 4 °C, were washed twice with PBS/5% FCS and were then incubated with FITC-conjugated goat anti-rat IgG for 1 h at 4 °C. After repeated washing with PBS/5% FCS, cells were fixed with 2% paraformaldehyde. Measurements were performed on an Epics® Coulter flow cytometer.

Confocal microscopy

The cellular distribution of CD44 on cultured MCT cells was determined by confocal microscopy. MCT cells were grown in DMEM/10% FBS medium on coverslips coated with type II collagen until subconfluent. Cells were then fixed in 4% paraformaldehyde and were blocked with 20 μM glycine and subsequently with 3% milk powder (w/v). For detection of intracellular CD44 cells were then permeabilized with 0.1% saponin (v/v). Rat anti-mouse mAb IM7.8.1 (5 μg/ml) in 3% milk powder was used to detect CD44. FITC-conjugated affinity-purified goat anti-rat IgG (Sigma, St Louis, MO) was used at 1:40 as secondary antibody.

CD44 expression was then assessed with a confocal laser scanning microscope (LSM 410, Zeiss, Germany) at magnifications of 630 × using the 488 nm line spectrum of a He-Ne laser source. Optical sections were obtained at focal steps of 28 or 56 nm. Stacks of 51 x-y sections were used to generate extended focus projections, x-z sections and 3-D reconstructions, using the Imaris® software (Bitplane, Zürich, Switzerland).

Shedding of CD44 from MCT cells

Shedding in response to crosslinking of anti-CD44 mAb was assessed by flow cytometry. Confluent MCT cells were detached and resuspended in DMEM/10% FBS and were then incubated with mAb IM7.8.1 at 10 μg/ml and crosslinked using anti-rat IgG for 5 h at 37 °C. Adhesion and clumping of MCT cells was prevented by shaking the tubes every hour. For detection of remaining cell surface CD44, cells were then stained with biotinylated KM114 mAb, using a streptavidin-R-phycoerythrin conjugate for detection (Sigma, St Louis, MO). Cells were then analysed by flow cytometry.

Soluble CD44 protein was also assayed in the supernatant of MCT cells by SDS–PAGE and Western blotting. Supernatants from control and from PMA-stimulated MCT (20 ng/ml) were size fractionated on a 10% SDS–PAGE gel under reducing and non-reducing conditions and blotted onto nitrocellulose. To detect soluble CD44 the membranes were then processed with a chemiluminescence detection system (Western-Star, Tropix, Bedford, MA). Membranes were blocked with casein and were incubated with the anti-CD44 mAb IM7.8.1. After washing, blots were incubated with alkaline phosphatase-conjugated goat anti-rat IgG

Table 1. Characteristics of anti-CD44 monoclonal antibodies

<table>
<thead>
<tr>
<th>Clone</th>
<th>Target</th>
<th>Properties</th>
<th>Source</th>
<th>Reference</th>
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<tbody>
<tr>
<td>IM7.8.1</td>
<td>CD44</td>
<td>Neutral</td>
<td>ATCC</td>
<td>[6]</td>
</tr>
<tr>
<td>KM81</td>
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<td>HA binding blocker</td>
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<td>IRAWB14.4</td>
<td>CD44</td>
<td>Induces HA binding</td>
<td>J. Lesley</td>
<td>[5]</td>
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CD44-mediated HA binding on TEC

(1:5000). The 1,2-dioxetane substrate (CDP-Star) was then added to the blots according to the instructions provided from the manufacturer. Membranes were exposed briefly (30 s to 5 min) to Kodak LS film and were then developed.

**HA binding to CD44 on MCT cells**

The HA binding capacity of MCT cells was assessed by flow cytometry. Confluent cells were detached and resuspended in PBS/5% FCS and incubated with HA-FITC at 1:800 for 1 h at 4°C. To control for non-specific binding cells were also incubated with FITC-labelled goat anti-rat IgG. The influence of anti-CD44 mAb on HA-binding was assessed by incubating cells with either purified mAb for 1 h at 4°C before staining with HA-FITC.

**Proliferation assays**

The influence of soluble HA on proliferation was assessed as follows: MCT cells were grown in 96-well plates (Nunc Intermed, Roskilde, DK) to confluence with DMEM/10% FBS. Cells were then rested for 24 h in DMEM/1% FBS. HA (500–1000 μg/ml) was then added and cells were incubated at 37°C for the indicated time. Proliferation was then assessed by [3H]thymidine incorporation, adding 1 μCi/well and harvesting the cells 18 h later with a Cambridge PHD cell harvester (Cambridge Technology, Cambridge, USA). [3H]thymidine incorporation was counted with a Kontron Betamatic V liquid scintillation counter.

**Results**

**Characterization of CD44 expression by MCT cells**

RNA was isolated from confluent MCT cells to detect the presence of transcripts encoding for the standard (CD44s) and the alternatively spliced isoforms of CD44 (CD44v) by RT-PCR and Southern blotting. Figure 1A shows that mRNA for CD44s was readily detected in the cell with no preference for apical, basal or lateral sides. Sections in the x-y direction indicated that most CD44 protein was membrane bound in MCT cells (Figure 2C, thin arrow), but a cytoplasmic fraction could also be detected in permeabilized cells (Figure 2C, dotted arrow). With the confocal images we also obtained evidence for shedding of CD44 by MCT cells. Figure 2D (3D reconstruction) shows CD44 retraction traces (arrow) and shedded remnants of cell membranes which are also CD44 positive (dotted arrow).

**Shedding of CD44 by MCT cells**

To characterize further the shedding of CD44 by MCT cells we used flow cytometry after crosslinking obtained by flow cytometry. Figure 2A shows that apical microvilli were strongly positive for CD44. The apical expression was confirmed by examining x-z (Figure 2B, bottom) and y-z sections (Figure 2B, upper right) which showed membrane bound CD44 around the cell with no preference for apical, basal or lateral sides. Sections in the x-y direction indicated that most of the CD44 protein was membrane bound in MCT cells (Figure 2C, thin arrow), but a cytoplasmic fraction could also be detected in permeabilized cells (Figure 2C, dotted arrow). With the confocal images we also obtained evidence for shedding of CD44 by MCT cells. Figure 2D (3D reconstruction) shows CD44 retraction traces (arrow) and shedded remnants of cell membranes which are also CD44 positive (dotted arrow).
CD44 with anti-CD44 mAbs. MCT cells were incubated with anti-CD44 mAb IM7.8.1 and bound mAb was crosslinked with anti-rat IgG. Figure 3A demonstrates that CD44 staining with biotinylated KM114 was intense (100%) in control cells (thick line). After crosslinking the mean fluorescence intensity decreased by 55% (thin line). Incubation of MCT cells with IM7.8.1 alone without crosslinking reduced the mean fluorescence intensity insignificantly (dotted line). Thus, crosslinking CD44 with IM7.8.1 and anti-rat IgG effectively decreased CD44 cell surface staining, suggesting shedding of CD44 by MCT cells via this mechanism.

To prove further that CD44 is shed by MCT cells we then performed SDS–PAGE and Western blotting to detect immunoreactive CD44 in the culture supernatant of MCT cells after stimulation with the phorbol ester PMA. Cells were grown to confluence and were then incubated with PMA for 48 h. Figure 3B shows that a protein around 80 kDa could be detected in the PMA-stimulated supernatants but not in non-stimulated MCT cell supernatants, demonstrating PMA-induced shedding of CD44 by MCT cells.

HA-binding capacity of MCT cells is modulated by mAb against CD44

To determine whether cell surface CD44 functions as a HA receptor on MCT cells we examined the HA binding capacity using a FITC-labelled HA probe. Incubation with FITC-labelled HA for 1 h showed moderate HA binding by unstimulated MCT cells (Figure 4). To exclude non-specific binding for HA-FITC, MCT cells were incubated with unlabelled HA at 10-fold excess before staining with HA-FITC. HA-FITC binding was completely abrogated by this procedure (data not shown).

As previously described, the anti-CD44 mAb KM81 inhibits the HA binding capacity in many cell types [7], whereas the activating anti-CD44 mAb IRAWB14.4 is able to induce or increase the HA binding capacity of lymphocytes [5,12]. To determine

![Fig. 2. Confocal microscopy of MCT cells stained for CD44. (A) Strong staining for CD44 on apical microvilli. (B) Cross-sections of two MCT cells. Upper left: x-y section, upper right: y-z section, bottom panel: x-z section. MCT cells express CD44 apically and basolaterally. (C) MCT cells were permeabilized with 0.1% saponin to detect intracellular CD44. Dotted arrow points to a cell with diffuse intracellular staining sparing the nucleus and more accentuated membrane staining. Thin arrow shows strong rim staining for CD44. (D) 3-D reconstruction of area where cells have retracted (arrow), demonstrating shedded remnants of cell membrane which are CD44 positive (dotted arrow) and CD44 retraction traces.](image-url)
Fig. 3. (A) Shedding of CD44 is induced with crosslinking CD44. MCT cells were incubated with anti-CD44 mAb IM7.8.1 and anti-rat IgG for 5 h and stained with biotinylated KM114 for remaining CD44. Shaded area: control (staining with secondary reagent); thick black line: control cells stained with biotinylated KM114 alone; dotted line: staining with biotinylated KM114 after incubation with IM7.8.1 without crosslinking; thin black line: staining with biotinylated KM114 after IM7.8.1 incubation with crosslinking. The fluorescence shift to the left after crosslinking of CD44 indicates shedding of CD44. (B) Detection of soluble CD44 in the supernatant of MCT cells by Western blotting. MCT cells were stimulated with PMA for 48 h in DMEM/1% FBS and the supernatant was harvested. Supernatants were run on a 10% SDS–PAGE gel using non-reducing conditions and blotted onto nitrocellulose. Soluble CD44 was detected using the IM7.8.1 mAb. Lane 1: control (48 h); lane 2: MCT cells stimulated with PMA for 48 h; lane 3: protein size marker (Da).

Fig. 4. Flow cytometric analysis for detection of HA binding to MCT cells. (A) The KM81 mAb inhibits HA binding on MCT cells. Cells were incubated with KM81 before incubation with FITC-labeled HA. (a) control (FITC-labeled anti-rat IgG only); (b) HA-FITC only; (c) KM81 at 10 μg/ml followed by HA-FITC; (d) KM81 at 50 μg/ml followed by HA-FITC. (B) The IRAWB14.4 mAb enhances HA binding on MCT cells. Cells were incubated with IRAWB14.4 before incubation with FITC-labeled HA. (a) control; (b) HA-FITC only; (c) IRAWB14.4 at 10 μg/ml followed by HA-FITC; (d) IRAWB14.4 at 50 μg/ml followed by HA-FITC.

DNA synthesis ([3H]thymidine incorporation) marked in a dose-dependent manner. Furthermore Figure 5B shows that a significant inhibitory effect of HA could also be demonstrated when MCT cells were grown on plate-bound HA (P < 0.005, t-test, five different experiments).

Discussion

CD44 is a versatile cell surface glycoprotein with numerous structural diversities and functional characteristics.
Fig. 5. Soluble and plate-bound HA inhibit the growth of MCT cells. Cells were grown in 96-well plates and proliferation was assessed by \[^{3}H\]thymidine incorporation. (A) Soluble HA when added for 24 h to confluent MCT cells dose-dependently inhibited proliferation (triplicate data from one of three representative experiments). (B) MCT cells grown on HA-coated (500 \(\mu\)g/ml) plates display reduced proliferation (data are pooled from five different experiments, each individual experiment was done in triplicate, \(P<0.005\)).

[2,13,14]. CD44 is expressed by renal proximal tubules in immune-mediated renal injury but not in normal kidneys [3,4,15,16]. Because the \textit{in vivo} role of enhanced tubular CD44 expression is not known we have examined a defined clonal TEC line (MCT cells) to characterize the expression pattern of CD44 and to examine the functional consequences of HA interaction with CD44 \textit{in vitro}. Here we show that cultured TEC express CD44 abundantly on the cell surface. CD44 can also be shed from the cell surface and is found in the supernatant of cultured MCT cells. We also show that CD44 on MCT cells functions as a HA receptor whose capacity to bind its ligand can be modulated with anti-CD44 antibodies. Furthermore we found that the binding of HA to CD44 inhibits cell proliferation markedly.

We chose the clonal cell line MCT to study the interaction of HA and CD44 on renal tubular epithelial cells because of many advantages compared to other cell lines or primary culture tubular cells. MCT cells display high constitutive expression of CD44s which is stable and not influenced by culturing conditions, while culture of primary tubular epithelial cells leads to upregulation of CD44 during the culturing process (unpublished results). Diseased renal tubular cells ([27], our unpublished results) and MCT cells have an apical and basolateral distribution of CD44 while transfected MDCK cells express CD44 only on the basolateral side [28]. MCT cells therefore reflect more closely the \textit{in vivo} situation. Also, renal tubular cell lines (e.g. C1 [29]) which need an extracellular matrix constituent to grow were not suitable for our experiments because the presence of collagen in the culture system could have influenced the results. Finally CD44 on MCT cells is not occupied by endogenous HA, thus allowing us to assess the interaction of HA and CD44 directly.

It is known that CD44 exists as a soluble molecule in addition to the cell surface form. Cleavage of CD44 from the cell surface by proteases or insertion of an additional alternatively spliced exon which introduces a premature stop codon (thereby creating a low molecular soluble CD44 isoform) have been implicated as possible mechanisms responsible for the shedding [17]. Our results demonstrate that MCT cells are capable of shedding CD44 after crosslinking or after stimulation by PMA. We can only speculate on the functional significance of CD44 shedding from epithelial cells such as TEC. Shedding has been described for various receptors involved in the regulation of the immune system, including adhesion molecules and cytokine receptors. A general function of shedding is the inhibition of interaction between receptor and ligand. Katoh \textit{et al.} have shown that soluble CD44 blocks binding of HA to cell bound CD44 [18]. It is possible that shedding of CD44 from TEC could also regulate the interaction of TEC with HA, thereby modulating cell growth and other processes such as cell differentiation.

The ability of CD44 to bind its natural ligand HA differs among cell lines. Three functional states can be distinguished with respect to HA binding: (i) non-binding; (ii) non-binding but inducible; and (iii) constitutively binding [2]. Monoclonal antibodies against CD44 can be used to modulate the binding for HA in various cells, presumably through changes in conformation of the CD44 molecule. The KM81 mAb for example blocks the binding of HA to CD44, whereas the IRAWB14.4 mAb induces strong HA binding [5,7]. MCT cells belong to the group of cells which bind HA constitutively. We found that the HA binding capacity could also be blocked with KM81 and stimulated with IRAWB14.4 in MCT cells. Although little is known
regarding the physiological stimuli that modulate HA binding on TEC and other cell types it is possible that biologically relevant stimuli such as growth factors or cytokines also influence the binding of HA.

Using MCT cells as a model system we found that soluble and plate-bound HA inhibit the growth of MCT cells. The role of CD44 and its ligand HA on cell growth has been investigated in several cell types of haematopoietic and non-haematopoietic origin and the relationship is complex. CD44 functions as a costimulatory molecule on T cells [13,19] but can also have a negative influence on T cell proliferation [20]. HA may either stimulate or inhibit endothelial cell and vascular smooth muscle cell proliferation, depending on the concentration and molecular weight of HA [21–24].

Our data suggest that CD44 could inhibit cell growth when interacting with its ligand HA on TEC. Dissociated proliferating cell nuclear antigen (PCNA) and CD44 expression in epithelial cells of colorectal neoplasms [25], or the progressive loss of CD44 gene expression in invasive bladder cancer [26] are other examples where epithelial CD44 seems to operate as an anti-proliferative molecule. We speculate that CD44 also has a growth inhibitory role on TEC in vivo. By limiting the regeneration rate of injured tubules CD44 could participate in the redifferentiation process in various settings of renal injury. Overshooting of tubular epithelial proliferation after injury could thereby be prevented.

In summary we have shown that MCT cells constitutively express the standard form of CD44 which is shed from the cell surface upon crosslinking of CD44 or after stimulation with PMA. CD44 functions as a receptor for HA on MCT cells and the binding capacity of CD44 for HA can be modulated by different anti-CD44 mAbs. The interaction of HA with its receptors significantly inhibits the proliferation of MCT cells by a mechanism which probably involves the CD44 protein. Since CD44 is expressed by injured proximal tubular epithelial cells we postulate that the interaction of HA with CD44 could regulate tubular epithelial cell growth and differentiation in vivo.

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CD44-mediated HA binding on TEC


