Hyaluronan is a prerequisite for ductal branching morphogenesis

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SUMMARY

Hyaluronan, a macromolecular carbohydrate polymer of the extracellular matrix is prominent early in embryogenesis, coinciding with rapid tissue growth. CD44, the predominant receptor for hyaluronan on vertebrate cells, is a variably expressed transmembrane glycoprotein. Mouse anterior prostate glands obtained at various postnatal time points were examined for the expression of hyaluronan and CD44. Reverse transcriptase polymerase chain reaction analysis was used to map the temporal regulation of specific CD44 variant isoforms. In each age group, hyaluronan was localized exclusively in the stromal matrix. Hyaluronan was greatly reduced in the later ages and was entirely absent around the developmentally quiescent proximal regions of the ducts. Early in prostate development, CD44 was prominent in the mesenchyme. However, in the later phases, CD44 expression became associated with membranes of epithelial cells. The role of hyaluronan-CD44 interactions in ductal branching morphogenesis was studied by serum-free organ culture of mouse anterior prostate. In the presence of optimal levels of testosterone, the organs underwent ductal branching morphogenesis. Treatment with either neutralizing anti-CD44 antibodies, hyaluronan hexasaccharides or the enzyme hyaluronidase inhibited androgen-stimulated ductal branching morphogenesis. These results are suggestive of the significant role played by hyaluronan-CD44 interactions in mediating androgen-induced prostatic growth and morphogenesis.

Key words: hyaluronan, CD44, prostate development, branching morphogenesis, mouse, epithelial-mesenchymal interaction

INTRODUCTION

Hyaluronan (HA) is a large polysaccharide in the extracellular matrix (ECM) with the repeating disaccharide unit of D-glucuronic acid and N-acetyl-D-glucosamine. HA, the principal glycosaminoglycan (GAG) in the ECM in a variety of mammalian tissues, is extremely hydrophilic and attracts large volumes of water of hydration. This results in expansion of the extracellular space and facilitates penetration of migrating cells (Toole, 1971; Toole and Gross, 1990).

The principal vertebrate receptor for HA is CD44, an integral plasma membrane glycoprotein that is involved in cell-cell and cell-matrix interaction (Trowbridge et al., 1982; Ahlo and Underhill, 1989). Sequence analysis of CD44 cDNAs from different species reveals homology in the extracellular amino terminus with the HA-binding domains of cartilage link and proteoglycan core proteins (Goldstein et al., 1989; Stamenkovic et al., 1989; Zhou et al., 1989; Aruffo et al., 1990; Bosworth et al., 1991; Gunthert et al., 1991).

CD44 is encoded by 20 exons; two constant regions, exons 1-5 and exons 16-20, and a 10 exon variable region ranging from exon 6 to 15 and referred to as v1-v10. These are variably expressed due to alternative splicing of the nuclear RNA resulting in numerous combinations of the variant exons which are expressed in different tissues (Screaton et al., 1993).

The prostate gland, under the influence of androgens, arises from solid epithelial anlagen emerging from the endodermal urogenital sinus below the bladder (Cunha et al., 1987). The prostatic buds elongate into the mesenchyme and undergo branching morphogenesis postnatally (Sugimura, 1986; Hayashi et al., 1991). Reciprocal mesenchymal-epithelial interactions are essential for prostatic development (Cunha et al., 1987, 1992). The urogenital mesenchyme (UGM) induces ductal morphogenesis, regulates epithelial proliferation and the expression of epithelial androgen receptors, and ultimately elicits prostate-specific secretory proteins (Cunha et al., 1980, 1983; Donjacour and Cunha, 1993). The epithelium of the developing prostate reciprocates by inducing the surrounding mesenchyme to differentiate into smooth muscle cells (Cunha et al., 1992).

We report here the first study of the expression of HA and CD44 in the developing prostate gland, exploring their spatiotemporal relationship. A serum-free mouse neonatal anterior prostate culture system has been adapted to elucidate the involvement of HA and HA-cell interactions in androgen-induced prostate ductal branching morphogenesis.

MATERIALS AND METHODS

Animal tissues

Male Balb/c mice from the Cancer Research Laboratory at the University of California (Berkeley, CA) were utilized in all experiments.

Immunoreagents

The monoclonal antibodies, KM201 and IM7.8, were generated in rats.
and directed against mouse CD44. KM-201 (IgG1; Trowbridge et al., 1982) was obtained as hybridoma (American Type Culture Collection, Rockville, MD) and purified from ammonium sulfate fractions of the hybridoma culture supernatants. IM7.8 (IgG2b; Miyake et al., 1990) was obtained from Pharmingen (San Diego, CA).

Preparation and characterization of HA-binding protein

The HA-binding protein (HABP) was prepared from bovine nasal cartilage (Pelfreeze, Rogers, AK) according to the method of Tengblad (1979).

Immunoblotting

Anterior prostates were removed from Balb/c mice, rinsed in PBS and homogenized in lysis buffer, 5 mM Hepes pH 7.4, 2 mM MgCl2, in a Dounce homogenizer. The large cellular debris were cleared by centrifugation at 6,500 g for 7 minutes. The membrane fraction was collected by centrifugation at 15,000 g for 7 minutes and resuspended in a modification of Laemmli’s buffer (Laemmli, 1970) without β-mercaptoethanol and without bromophenol blue. The protein content was determined and 15 μg of each sample was resolved on 10% SDS-polyacrylamide gel. The protein was transblotted onto nitrocellulose membrane (Bioread, Hercules, CA). The membranes were blocked with 5% non-fat milk in PBS and 0.2% Tween 20 for 30 minutes. The protein CD44 was detected by incubation with KM201 at a dilution of 1:100 in blocking solution, for 60 minutes at room temperature. The blots were rinsed with three changes of TBST (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Tween-20) over 30 minutes, and followed by incubation with biotinylated secondary anti-rat IgG diluted 1:200 in TBST, for 60 minutes, and further rinsing in TBST. The signal was detected with a horseradish peroxidase detection kit (Vector, Burlingame, CA).

HA histochemistry

Mouse anterior prostates were fixed with paraformaldehyde, embedded in paraffin, sectioned (6 μ) and mounted on polylysine-coated glass slides for histological staining with HABP. The specificity of the staining was determined by two methods. (1) Predigestion of sections with Streptomyces hyaluroni-dase. 100 TRU/ml, 0.1 M sodium formate buffer, 0.15 M NaCl, 1 mg/ml BSA, 0.1% Triton X-100, pH 3.7, overnight at 37°C. (2) The biotinylated HABP was diluted 1:100 with HABP-buffer solution (0.25 M sodium phosphate buffer, pH 7.0 containing 1.5 M NaCl, 0.3 M guanidine-HCl, 0.08% BSA and 0.02% Na2HPO4, pH 7.0) and combined with a solution of 0.1 mg/ml HA (Sigma, St. Louis, Mo), prior to application to the sections. The control slides, together with the experimental slides, were incubated with 3% BSA at 37°C for 30 minutes. After rinsing with PBS-CMF, 0.3 ml of the biotinylated HABP, diluted 1:100 in HABP-buffer was added to each experimental and control slide. All slides were incubated overnight at 4°C. The slides were rinsed in PBS-CMF and transferred to 0.6% H2O2 in methanol at room temperature. After rinsing with PBS-CMF, staining was achieved using the substrate 3,3'-diaminobenzidine ( Peroxidase Substrate Kit, DAB SK 4100, Vector, Burlingame, CA) for 5 minutes, followed by a rinse with ddH2O. Slides were counterstained for 15 minutes in 0.25% methyl green, rinsed in ddH2O and dipped sequentially in 70%, 95% and 100% ethanol, and xylene.

CD44 immunohistochemistry

The sections were deparaffinized and hydrated following standard procedures. The slides were then placed in 10 mM citrate buffer, pH 5.8 and incubated at 70°C of full microwave power for 15 minutes. Endogenous peroxidase activity was blocked by incubating for 30 minutes in 2 ml of 0.6% H2O2 in methanol. After rinsing in PBS-CMF for 20 minutes, non-specific binding sites were blocked by incubation with 5% normal goat serum in filtered PBS-CMF for 30 minutes, at 37°C. The primary antibody used was the unconjugated rat anti-mouse CD44 mAb, IM7.8 IgG2b (Pharmingen). The antibody, diluted 1:150 in PBS-CMF containing 5% normal goat serum, was incubated for 1 hour at 37°C, then overnight at 4°C. The secondary antibody, biotinylated goat anti-mouse IgG diluted in 5% normal goat serum, was incubated for 45 minutes at room temperature. Each one of the above steps was followed by three 5 minute washes in PBS-CMF. 0.1% Tween 20. Bound antibodies were visualized using an avidin-biotin horseradish peroxidase detection system.

All histochemistry was documented by photography using an Olympus Vanox AHTB3 Microscope (Olympus Co., Woodbury, NY), with an integrated Olympus C-35AD-4 camera and Kodak Gold Plus film (Eastman Kodak Co., Rochester, NY).

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Tissue was dissected from the animals and the mRNA extracted using RNA Track (Biotec). The mRNA was used as a template for the first strand synthesis of cDNA in a reaction mixture containing 1000 U Moloney murine leukemia virus reverse transcriptase, 50 μM random hexamer primer, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM dithiothreitol, 0.3 mM MgCl2, 0.5 mM each of dGTP, dATP, dTTP and dCTP in 60 μl. The reactions were carried out at 37°C for 90 minutes and then diluted 1:3 in water pretreated with DEPC.

Oligonucleotide primers were diluted to 40 μM in water. For RT-PCR, a modified hot start method was used. 3-5 μl of cDNA were added to 30 μl of a master mix containing 0.8 μM of each primer, 17 mM Tris-HCl pH 8.3 and 80 mM KCl. Water was added to a final volume of 40 μl. All reactions were overlaid with a light mineral oil and heated to 99°C. After 10 minutes, the reactions were cooled to 94°C and a 10 μl volume containing 7 mM MgCl2, 1 mM each of dATP, dTTP, dGTP and dCTP, and 1.2 U Taq polymerase added. The products of RT-PCR were separated on 2% agarose and visualized by UV light.

The primers used for amplification were as follows:

Forward variant 0  5'-GCAAAATTCCTTATCCGGAGCACCTTG-GCC-3'
Forward variant 7  5'-GCCCAACAAACACATCCAAGTCA-3'
Reverse variant 11  5'-GCAGTGGGTAAGGGTGTAC-3'
Reverse variant 11  5'-CGGGGTACCTGCCCATGACACCA-GTCG-3'

Hyaluronic acid ELISA

A competitive ELISA was used to measure HA. HA (0.2 mg/ml) (ICN, Irvine CA) was bonded to 96-well microtiter plates (Corning Glassworks, Corning, NY). This was achieved by 24 hours incubation with 0.184 mg/ml of N-hydroxysulfosuccinimide and 1.23 mg/ml of 1-ethyl-3-dimethyl-anopropyl carbodiimide, at 4°C. Non-specific binding was blocked by incubation with 300 μl/well of 0.6% nonfat milk, at 37°C for 30 minutes. Prostate extract samples were incubated at 37°C for 1 hour, with an equal volume of biotinylated HABP. The prostate extract-HABP mixture was added to the microtiter plate in aliquots of 100 μl and incubated at 37°C for 1 hour. Rinsing with PBS-0.5% Tween 20 washed away the HABP bound to the prostate extract HA. The excess biotinylated HABP bound to the HA on the microtiter plate. A standard HA concentration curve was generated for each ELISA plate. The HABP bound to the plate was detected by coupling an avidin-biotin-peroxidase complex (Vector, Burlingame, CA) to an ω-phenylenediamine substrate reaction. The absorbance at 492 nm was read on a Titertek Multiskan Plus spectrophotometer (Lab Systems, Helsinki, Finland). All readings were done in triplicate.

Preparation of HA hexasaccharide

Hyaluronic acid hexasaccharides were prepared using the technique described by Banerjee and Toole (1991). The chromatographic fractions with a uronic acid (Bitter and Muir, 1962) to terminal hex-
osamine (Reissig et al., 1955) ratio of between 2.9 and 3.1 were pooled and used in experiments requiring HA hexamers.

Preparation of hyaluronidases

Hyaluronidase was purified from human plasma as described by Frost et al. (1997). Streptomyces hyaluronidase (Calbiochem, La Jolla, CA), an enzyme that specifically degrades HA, but does not adversely affect embryonic viability (Morriss-Kay et al., 1986) was preabsorbed with agarose-ovomucoid (Sigma, St. Louis, MO), 1 U/g agarose, overnight at 4°C, to strip the enzyme of contaminating proteases. Agarose was precipitated by centrifugation. Hyaluronidase activity was characterized by hyaluronidase ELISA-like assay as described by Stern and Stern (1992) and HA-substrate gel electrophoresis (Guntenhon et al., 1992).

Organ culture

Mouse anterior prostates (AP) were maintained in an organ culture (1992) and HA-substrate gel electrophoresis (Guntenhon et al., 1992). The animals were killed by decapitation and the genital tract of each animal removed. Further dissection to isolate the anterior prostate (AP) was performed in a watchmaker’s depression slide, with a Dage-MTI CCD-72 TV camera (Olympus Co., Woodbury, NY), interfaced with a Macintosh Quadra 800 computer (Apple, Cupertino, CA), and processed by image analysis software (NIH Image 1.41, Bethesda, MA). Epithelial area and perimeter were calculated from computer-generated binary images of the glands. Node number, a count of the number of branch points, was defined as the number of internal connections between three or more links in a computer-generated skeleton of the AP epithelial outline. This parameter was used to determine morphological complexity of each organ’s epithelial shape. The complexity of the shape of the developing AP, form factor, was calculated as the ratio of area to perimeter (Form factor = area/perimeter²). The inverse of form factor was determined in order to achieve a direct correlation between morphological complexity and the value determined as form factor (1/form factor).

Protein determination

For protein determination, the BioRad Microassay Kit (BioRad Laboratories, Richmond, CA) was utilized with microtiter plates (Costar, Cambridge, MA).

Statistical analysis

Descriptive statistics on mean and standard deviation were determined for all the quantitative parameters and displayed as histograms. Comparison of epithelial area, epithelial perimeter, node number, and inverse form factor were made by analysis of variance (ANOVA) and intergroup difference determined by Fisher’s multiple comparison test, with the level of significance set at P<0.05.

RESULTS

Detection of hyaluronan in developing prostate gland by histochemistry

HA was localized in the prostate tissues at different stages of development using the biotinylated HABP as a histochemical stain (Fig. 1). During the early stages of development at day 0 (Fig. 1A), HA was a prominent component of the stromal connective tissue, with no specific spatial predilection. There was no HA associated with epithelial components. At 10 and 15 days postnatal, the intensity of HA staining in the stroma had decreased (Fig. 1B,C). HA staining was significantly more intense around the growing tips of the buds undergoing branching morphogenesis and less pronounced in the developmentally quiescent regions of the ducts. These trends continued as development progressed through 30 days postnatal (Fig. 1D), with the intensity of HA staining decreasing even further.

Detection of CD44 protein by immunohistochemistry

To determine the localization of CD44, during prostatic development, sections were stained with anti-CD44 mAb (Fig. 2). Localization was dependent on the developmental stage. In sections from prostate at 0 days (Fig. 2A), CD44 mAb stained the mesenchyme. The 0 day time point coincided with the initiation of ductal branching which, in the mouse prostate, is a postnatal process. No expression was observed in the epithelium or associated with the basal lamina. At 10 days (Fig. 2B), CD44 was localized to the basolateral cell membranes of the epithelium, with no evidence of mesenchymal expression. The staining was generally uniform throughout the epithelium. This pattern was maintained through day 15 and CD44 became increasingly focalised to the developing distal tips of the epithelial ducts (Fig. 2C). No immunostaining was evident at 30 days (Fig. 2D). Background staining as determined by substituting biotinylated IgG for the anti-CD44 mAb was consistently negative (not shown).

Immunoblot analysis of CD44 protein expression

The different isoforms of CD44 present in the developing prostate from day 0 to 30 days were examined by western blotting with the KM201 mAb (Fig. 3). The predominant CD44 species expressed was detected at 85 kDa and corresponds to the CD44S isoform. The relative abundance of the 85 kDa form remained unchanged throughout the period studied. At 5 days, CD44 forms of approximately 116, and 160 kDa were detected, in addition to the 85 kDa form. This pattern of expression was maintained through day 10 and 15; however, at 30 days and beyond, the higher molecular weight isoforms were of low abundance.

RT-PCR analysis of CD44 splice variant expression

Reverse transcription polymerase chain reaction (RT-PCR) analysis was carried out to identify the pattern of CD44 splice variants expressed at the various time points in the developing
anterior prostate. mRNA isolated from the anterior prostate was used to generate cDNAs, which were subsequently used as templates for PCR using amplimers specific for both constitutive and alternatively spliced exons (Fig. 4). Amplification and electrophoretic separation demonstrated PCR products, the sizes of which were consistent with the presence of mRNA encoding both CD44S, and alternatively spliced isoforms of CD44, in the anterior prostate (Fig. 5). cDNA amplified with primers FV0 and RV11 produced a product of 112 bp representative of CD44S, in which all the variable exons have been removed by alternative splicing. This form of CD44 was consistently expressed during the entire period of development that was studied. Amplification by primers FV7 and RV8 produced a 161 bp product. The variants containing exon 7 displayed a specific window of expression confined to between 5 and 10 days.

**Perturbation of developing prostate ductal branching morphogenesis**

(A) Perturbation with antibodies against CD44

The anterior prostates (AP) of 0-day-old mice were cultured for 6 days. The four parameters assayed as a measure of ductal branching morphogenesis were epithelial area, epithelial perimeter, node number and form factor. Form factor is a measure of shape complexity derived from the perimeter and area; the simplest shape being the circle with a form factor of one. Since form factor decreases as a shape achieves greater complexity, this parameter was computed as inverse form factor. Node number describes the number of branching points in a duct. At day 0, the AP was a simple unbranched epithelial anlagen surrounded by mesenchyme. At 6 days of culture in the presence of testosterone, the gland was significantly enlarged. In culture, the AP is free of the influence of the surrounding seminal vesicle and its associated tissues. This, in addition to the limitations imposed by the organ culture system, result in the organ approximating a 2-dimensional pattern.

To determine the significance of CD44-mediated interactions in AP ductal branching morphogenesis, mAb KM201 was added to 0-day-old Balb/c mouse APs cultured with testosterone, and the parameters described above were analyzed. Anti-CD44 mAb inhibited testosterone-induced ductal branching morphogenesis (Figs 5, 6). Addition of rat IgG of the same class as mAb KM201, but not recognizing CD44, had no effect on branching morphogenesis.

In APs cultured with 50 ng/ml anti-CD44 mAb plus testosterone, the epithelial area, epithelial perimeter, inverse form factor and node number were 54.8% (P<0.0001), 51.7% (P<0.0001), 48.9% (P=0.006), and 71.4% (P=0.0139) respectively, of the level achieved by optimum testosterone alone. At an anti-CD44 mAb concentration of 100 ng/ml, epithelial area was 37% (P<0.0001), epithelial perimeter 33.9% (P<0.0001), 1/FF 30.9% (P=0.0017), and number of nodes 15.2% (P<0.0001) of the values achieved in APs cultured with testosterone alone. Therefore, anti-CD44 mAb significantly inhibited ductal branching morphogenesis. In cultures without testosterone, the anti-CD44 mAb did not further reduce the epithelial parameters.

(B) Perturbation with hyaluronan hexamer

The results reported above suggest that interaction between HA and HA-binding protein is necessary for AP ductal branching morphogenesis. In that case, other reagents that disrupt interaction between HA and HA-binding protein, in addition to anti-CD44 antibodies, should affect branching. HA hexamer competitively inhibits interaction between polymeric HA and cell surface HA-binding proteins, while not competing for interaction with interstitial HA-binding proteins (Underhill and Toole 1979; Yamagata et al., 1986).

HA hexamer was inhibitory to AP ductal branching morphogenesis (Figs 7, 8). At 80 ng/ml, partial inhibition was attained; node number decreased to 64.7% (P<0.0001), inverse form factor to 96.8% (NS), epithelial perimeter to 84.3% (NS), and epithelial area to 73.4% (P=0.0118) compared to optimal testosterone-induced branching. At a higher concentration of HA hexamer (150 ng/ml), the same parameters were further reduced: epithelial area to 55.7% (P=0.0003), epithelial perimeter to 50.8% (P=0.0010), 1/FF to 46.4% (P=0.004) and

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node number to 11.8% (P<0.0001) of the optimal testosterone-induced levels. There was significant inhibition of ductal branching morphogenesis at 150 ng/ml. No effect was obtained by equivalent concentrations of Na uronate and N-acetylglucosamine (not shown).

On withdrawal of the hexamer, the APs resumed growth. This observation eliminates toxicity as the cause of the inhibitory effect.

Fig. 3. Western analysis of CD44 expressed by the developing anterior prostate at various ages of development. The predominant 85 kDa species (CD44S) was the only CD44 variant detected at day 0 (lane 1), and remained unchanged throughout the ages studied. At 5 days (lane 2), CD44 isoforms of approximately 116 and 160 kDa were also detected. This was maintained through days 10 and 15 (lanes 3 and 4, respectively).

(C) Perturbation with hyaluronidases
Treatment of cultured APs with 20 and 100 TRU/ml hyaluronidase (both plasma and Streptomyces), with or without testosterone, effectively removed HA from the developing organs (Figs 9-12).

Plasma hyaluronidase distorted the pattern of branching (Figs 9, 10). At 20 TRU/ml, epithelial area was 72.3%

Fig. 4. Detection of transcripts for CD44S (A), CD44 variants 7 and 8 (B), and control transcripts TAP 2 (C) at various ages in the development of the mouse anterior prostate (See Materials and Methods for techniques). Lane 1, 0 day; lane 2, 5 days; lane 3, 10 days; lane 4, 30 days; lane 5, 45 days.
cultured for 6 days in serum-free medium. (P=0.0024), epithelial perimeter 69.3% (P<0.0001), 1/FF 66.4 (P<0.0001), and node number 17.3% (P<0.0001) compared to the equivalent measures in cultures with testosterone alone. At a concentration of 100 TRU/ml, epithelial area was 40.1% (P<0.0001), epithelial perimeter 42.3% (P<0.0001), 1/FF 44.7% (P<0.0001) and node number was 13.0% (P<0.0001) of the optimal measures.

Streptomyces hyaluronidase effected a similar pattern of distortion (Figs 11, 12). The pattern of branching at 20 TRU/ml; epithelial area was 71.5% (P=0.0004), epithelial perimeter 48.6% (P<0.0001), 1/FF 32.3% (P=0.0002) and node number 86.4% (P=0.011) in relation to the values achieved when cultured under optimal conditions (testosterone without antibodies). At 100 TRU/ml epithelial area was 31.1% (P<0.0001), epithelial perimeter 37.8% (P<0.0001), 1/FF 25.5% (P<0.0001), and node number 9.1% (P<0.0001) of the optimal levels.

The growth and development of APs cultured with hyaluronidase in the absence of testosterone was equivalent to APs grown in the absence of testosterone. Significantly, treatment with hyaluronidase did not induce epithelial reduction to levels below those observed in the absence of testosterone.

**DISCUSSION**

Hyaluronan (HA) has been shown to play a fundamental role in cell differentiation and motility (Laurent and Fraser, 1992; Sherman et al., 1994). HA influences these biological phenomena by interacting with specific HA-binding cell-surface receptors, which include CD44 (Aruffo et al., 1990; Goldstein et al., 1989; Kaufmann et al., 1995; Lesley et al., 1993; Miyake et al., 1990; Zhang et al., 1995), CD38 (Nishina et al., 1994), I-CAM (McCourt et al., 1994) and receptor for HA-mediated motility (RHAMM) (Hoare et al., 1992; Turley et al., 1993; Yang et al., 1992). A compelling HA-binding motif that is common to all HA-binding proteins has recently been described (Yang et al., 1994). However the HA-binding motif does not account for all the HA-binding properties of these proteins (Hoare et al., 1993; Toole, 1990) and this is suggestive of a role for the three-dimensional configuration of the receptor.

Sequence analyses of CD44 indicate that the extracellular
domain of CD44 contains homologies to the HA-binding region of both aggrecan and cartilage link protein (Goldstein et al., 1989; Stamenkovic et al., 1989). The murine CD44 receptor for HA has been described (Miyake et al., 1990; Aruffo et al., 1990). Indeed Aruffo et al. (1990) confirmed HA to be the CD44 ligand by attenuating lymph node tissue reactivity to a soluble CD44 receptor immunoglobulin fusion protein following hyaluronidase treatment of the lymph node. The many important biological processes associated with HA, particularly in early embryology, tumor invasiveness, and tissue repair and remodeling, signify that the relationship between CD44 and HA is of special importance. It has become apparent that several different isoforms may be generated from the CD44 gene by alternative RNA splicing. Some of these alternative isoforms have distinct properties and ligands other than HA.

The mAbs KM201 and IM7.8 recognize all the isoforms of CD44. In this study, these antibodies were used to demonstrate that the HA-binding form of CD44 was the predominant isoform expressed in the developing anterior prostate throughout the time points studied, 0 to 30 days. Isoforms with higher molecular mass were observed coinciding with the initiation of ductal branching. This observation was reinforced by RT-PCR analysis using amplimers specific for several isoforms of CD44. This demonstrated that, at specific time points, developing anterior prostate expressed, in addition to CD44S, alternatively spliced isoforms of CD44. These time points were coincident with the detection of high relative molecular mass CD44 forms by immunoblotting.

The influence of an HA-rich ECM on resident cells is a product of both the hydration properties of HA (Comper and Laurent, 1978), and the interaction between HA and cell surface HA-binding proteins (Toole, 1990). This influences cellular processes critical to embryogenesis and morphogenesis, viz. cell shape, cell migration and cell proliferation. CD44-HA interactions influence cellular function by a number of different mechanisms. In the developing hair follicle, a close correlation between the expression of CD44 and the absence of HA, both spatially and temporally, is demonstrated (Underhill, 1993). This suggests that the expression of CD44 by the inductive mesenchymal cells results in removal of HA. Similar observations have been made in other systems such as the developing lung, tooth and liver (Underhill, 1993; Underhill et al., 1993). This phenomenon is reinforced by studies demonstrating that macrophages and fibroblasts that express CD44 do degrade HA (Culty et al., 1990). Apparently, HA binds to the cell surface and is taken up into the cell and degraded by lysosomal enzymes. In contrast to this, in the epidermis, the patterns of expression of CD44 and HA are directly correlated. Both CD44 and HA are expressed in the early eriderm, and in the stratum spinosum of the mature epidermis (Ahlo et al., 1989; Wang et al., 1992). A critical difference in the epidermis compared to the mesenchymal cells, is the expression by the keratinocytes of the 180 kDa isoform...
be involved in functions other than the binding and degradation of HA.

The synthesis of HA is related directly to the rate of cell proliferation (Matuoka et al., 1987). In the developing prostate, the rate of cell proliferation is particularly high in the distal tips of the developing ducts (Sugimura et al., 1986). Our histological data suggest that HA is associated with the proliferating prostatic ducts. The prostate predominantly expresses the 85 kDa CD44S form at all stages of development. This is the form that is characterized as the major HA receptor (Aruffo et al., 1990; Lesly et al., 1990; Miyake et al., 1990; Stamenkovic et al., 1989). Furthermore, there is an obvious temporal and spatial regulation of CD44 expression directly correlated with the expression of HA circumscribing the distal ductal tips. The segregation of CD44 expression at 10, 15 and 20 days to the apical and lateral aspects of the ductal epithelium is suggestive of a role for CD44-HA adhesion in cell-matrix interactions. The results of the CD44 immunoblots, CD44 immunohistochemistry and the RT-PCR analysis, taken together in conjunction with previous studies, suggests a function for CD44 in the process of prostatic branching morphogenesis. The CD44S form regulates levels of HA by receptor-mediated internalization, as described by Culty et al. (1992) and Hall and Miyake (1992). Furthermore, cell-surface-receptor-mediated attachment to HA is directly implicated in morphogenesis (Banerjee and Toole, 1992).

The expression of high relative molecular mass CD44 variants within a precise window of development suggests that these variants are involved in the ‘invasion’ of the epithelial ducts into the mesenchyme. This would fit into the paradigm that metastatic invasion during malignancy reflects a recapitulation of the molecular mechanisms involved in embryogenesis and morphogenesis. These CD44 variants have been shown to be of major importance for tumor dissemination in

Fig. 9. Representative images of mouse APs cultured for 6 days in serum-free medium. (A) Day 0, before culture; (B) without testosterone; (C) with testosterone (10^{-8} M); (D) with testosterone + 20 TRU/ml Streptomyces hyaluronidase; (e) with testosterone (10^{-8} M) +100 TRU/ml Streptomyces hyaluronidase.

Fig. 10. Effect of Streptomyces hyaluronidase on mouse prostate APs following 6 days incubation in serum-free medium. Four parameters of epithelial morphogenesis were assessed: (A) epithelial area, (B) epithelial perimeter, (C) number of nodes, (D) inverse form factor (1/FF). (T, testosterone; S. h’ase, Streptomyces hyaluronidase).
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rat pancreas carcinoma (Gunthert et al., 1991; Seiter et al., 1993), in human colon cancer (Mulder et al., 1994) and mammary carcinoma (Kaufman et al., 1995).

The development of AP using the serum-free organ culture system was androgen dependent and ductal branching morphogenesis in this model replicated normal development of the prostate. This study examines the function of HA in ductal growth and branching morphogenesis by comparing the effects of various anti-HA-cell interaction factors. For the first time, we can effectively pose the question: Is HA necessary for ductal growth and branching morphogenesis of the prostate? The novel contribution of this study lies in the application, for the first time, of computer-based morphometrics to analyze the effects of HA in a serum-free culture system of the developing neonatal prostate. The parameters measured, epithelial area, epithelial perimeter, node number and epithelial form factor (a parameter for shape complexity) are sufficient for the quantification of growth and ductal branching morphogenesis.

HA oligomer and CD44 mAb both inhibit branching morphogenesis of the prostate. The inhibition was reversible. The mechanism by which CD44 mAb inhibits ductal branching morphogenesis is presumably due to its specific binding to CD44, which in turn would block interaction of endogenous HA with the receptor CD44. Previous studies have shown that antibodies to HA-binding proteins block binding of exogenous HA to both soluble and membrane-bound HA-binding proteins (Banerjee and Toole, 1991; Knudson and Knudson, 1991; Yu et al., 1992).

The HA hexamer was utilized because of its ability to competitively block HA-cell-surface-binding protein interactions (Underhill and Toole, 1979). The precise mode of action of the oligomer in inhibiting ductal branching has not been explored intensively; however, it is suggested that incorporation of the oligomer into the organ culture medium leads to disruption of HA-CD44 interactions. The conclusion from this observation is that treatment with the HA oligomer results in interference with CD44-HA interaction, leading to inhibition in branching morphogenesis. The results described above suggest that HA-CD44 interaction is necessary for prostatic ductal branching morphogenesis.

**Fig. 11.** Representative images of mouse APs cultured for 6 days in serum-free medium. (A) Day 0, before culture; (B) without testosterone; (C) with testosterone (10^{-8} M); (D) with testosterone + 20 TRU/ml plasma hyaluronidase; (E) with testosterone (10^{-8} M) +100 TRU/ml plasma hyaluronidase.

**Fig. 12.** Effect of plasma hyaluronidase on mouse prostate APs following 6 days incubation in serum-free medium. Four parameters of epithelial morphogenesis were assessed: (A) epithelial area, (B) epithelial perimeter, (C) number of nodes, (D) inverse form factor (1/FF). (T, testosterone; P. h'ase, Plasma hyaluronidase).
CD44 is expressed and is functional during the early postnatal period of prostatic development, and plays a significant role in the development of the mouse prostate. Ductal branching morphogenesis is certainly a very complex process. It is possible that variations in this development system might create the slight differences observed between the various anti-CD44-HA interaction reagents. Further studies are required to determine precisely the dose-response effectiveness of both the antibodies and the hexasaccharide in the commitment of the UGS into prostatic differentiation and in bud formation.

Cultured AP organs were also treated with either Streptomyces hyaluronidase or plasma hyaluronidase. The experiments in this phase were designed to explore the effect of degrading HA by hyaluronidase on androgen-induced prostate development. In both cases, hyaluronidase treatment of cultured AP effectively removed HA from the mesenchyme. HA degradation resulted in significant attenuation of branching. These findings provide insights into the essential function of HA accumulation in the ontogeny of normal prostatic ductal branching morphogenesis. The presence of HA in the developing prostate extracellular matrix is necessary for ductal branching morphogenesis, and absence is associated with substantial alterations in the branching. The observation that branching did not take place after treatment with hyaluronidase is consistent, with observations in other biological systems, principally in the developing endocardial cushions (Baldwin et al., 1994; Bernanke and Markwald, 1982). It has been observed that incorporation of HA to the collagen gel bioassay of endocardial cushion formation creates a two-fold increase in the epithelial-mesenchymal transformation of atrioventricular canal endothelial cells (Barnake and Markwald, 1982), while treatment with hyaluronidase had an inhibitory effect on epithelial-mesenchymal transformation in the same model (Krug et al., 1985).

Here we demonstrate the expression of specific CD44 isoforms in prostatic epithelium during periods in which the epithelium is undergoing mesenchymal cell differentiation. We interpret our results to mean that prostate morphogenesis is mediated, in part, through modulation of CD44-HA interactions. The results of our studies indicate that disrupting HA-CD44 interaction is a potent way of inhibiting testosterone-induced prostatic ductal branching morphogenesis and epithelial growth. At low concentrations of anti-CD44 mAbs and the HA hexasaccharide, with optimal testosterone, ductal branching morphogenesis was distorted, but epithelial area was maintained at a level comparable to that of positive controls. At high concentrations of anti-CD44 mAbs and HA hexasaccharide in the presence of testosterone, ductal branching morphogenesis was significantly inhibited. However, epithelial area was comparable to that of glands at the initiation of culture. This suggests that interrupting the HA-cell interaction did not induce loss of epithelial cells as might be triggered by cell death, although testosterone-induced increase in epithelial area was completely inhibited.

Our results show that CD44-mediated cell adhesion to mesenchymal HA is involved in the regulation of ductal branching morphogenesis in the mouse prostate. Absence of effective CD44-HA interaction distorts the delicately controlled regulation, resulting in significant changes in the ductal branching pattern. Mapping of the precise mechanisms through which this specific interaction functions is underway and this will elucidate some aspects of the epithelial-mesenchymal controls in parenchymal morphogenesis.

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