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Activin A is an endogenous inhibitor of ureteric bud outgrowth from the Wolffian duct

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Abstract

Development of metanephric kidney begins with ureteric bud outgrowth from the Wolffian duct (WD). GDNF is believed to be a crucial positive signal in the budding process, but the negative regulation of this process remains unclear. Here, we examined the role of activin A, a member of TGF-β family, in bud formation using an in vitro WD culture system. When cultured with the surrounding mesonephros, WDs formed many ectopic buds in response to GDNF. While the activin signaling pathway is normally active along the non-budding WD (as measured by expression of activin A and phospho-Smad2/3), activin A was absent and phospho-Smad2/3 was undetectable in the ectopic buds induced by GDNF. To examine the role of activin A in bud formation, we attempted to inactivate activin action. Interestingly, the addition of neutralizing anti-activin A antibody potentiated GDNF action. To further clarify the role of activin A, we also tested the effect of activin blockade on the WD cultured in the absence of mesonephros. WDs without mesonephros did not form ectopic buds even in the presence of GDNF. In contrast, blockade of activin action with a variety of agents acting through different mechanisms (natural antagonist, neutralizing antibodies, siRNA) enabled GDNF to induce ectopic buds. Inhibition of GDNF-induced bud formation by activin A was accompanied by inhibition of cell proliferation, reduced expression of Pax-2, and decreased phosphorylation of PI3-kinase and MAP kinase in the WD. Our data suggest that activin A is an endogenous inhibitor of bud formation and that cancellation of activin A autocrine action may be critical for the initiation of this process.

Keywords: Wolffian duct; Activin; Ureteric bud outgrowth; Smad; BMP4; Mesonephros; Ectopic bud; Nephrogenesis; Autocrine

Introduction

The embryonic kidney forms through reciprocal interactions between the metanephric mesenchyme and the ureteric bud (UB) (Saxén, 1987). The metanephric mesenchyme localizes at the caudal end of the intermediate mesoderm and induces the UB from the neighboring Wolffian duct (WD). The invading UB induces the metanephric mesenchyme to aggregate and epithelialize to form renal vesicles, which undergoes complex morphogenetic changes to form the mature nephron, the functional unit of the kidney (Shah et al., 2004). Ectopic budding of the UB from the WD leads to many congenital anomalies of the kidney and urinary tract such as hypoplastic kidney, ectopia of the ureterovesical orifice, urinary outflow obstruction, and/or reflux (Ichikawa et al., 2002; Pohl et al., 2002). Elucidation of the budding mechanism is essential for understanding how these abnormalities of the kidney and urinary tract develop. However, the mechanisms behind this process have only been partly elucidated. It is currently thought that UB emerges from the WD in response to glial-cell-derived neurotrophic growth factor (GDNF) produced by metanephric mesenchyme (Shakya et al., 2005). Recent evidence indicates that GDNF action is tightly regulated by several factors or signaling systems during the UB outgrowth from the WD. For example, mice lacking Sprouty1 (the receptor tyrosine kinase antagonist) have supernumerary ureteric buds. This phenotype was caused by enhanced sensitivity of the WD to GDNF, resulting in the development of multiple ureters and multiplex kidneys (Basson et al., 2005). In Slit/Robo knockout mice, it was also

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shown that the expression of GDNF was inappropriately maintained in anterior nephrogenic mesenchyme, resulting in the development of supernumerary ureteric buds (Grieshammer et al., 2004). These data suggest that negative regulatory systems are also operative in this process.

Activins are multifunctional cytokines structurally related to transforming growth factor (TGF)-β (de Caestecker, 2004). Like other members of the TGF-β superfamily, activins modulate cell proliferation, induce differentiation, and are involved in organogenesis and tissue remodeling (Maeshima et al., 2001a; Welt et al., 2002). Activins are dimeric proteins, and subunits of activin are expressed in various organs (ten Dijke and Hill, 2004). An important modulator of activin action is follistatin (Nakamura et al., 1990), which specifically binds to activins and related ligands with high affinity and blocks their action (Nakamura et al., 1990; Shimonaka et al., 1991). In general, activins exert their actions as autocrine or paracrine factors (Welt et al., 2002). Among activins, activin A is expressed in the kidney (Tuuri et al., 1994). Follistatin is also expressed in this organ abundantly (Shimasaki et al., 1989). Activin A blocks branching morphogenesis of the developing kidney collecting system (Bush et al., 2004; Maeshima et al., 2003; Ritvos et al., 1995) and also inhibits tubulogenesis in cultured renal epithelia cells (Maeshima et al., 2000b) as do TGF-βs (Sakurai and Nigam, 1997; Santos and Nigam, 1993), raising the possibility of a potential role for activin A as one of the negative regulators of kidney development.

Here, we demonstrate the importance of activin A in UB emergence from the WD. When cultured in the absence of mesonephros, WDs did not form ectopic buds in the presence of GDNF. However, perturbation of activin action with a number of mechanistically distinct agents (antagonist, neutralizing antibodies, siRNA) allowed GDNF to induce ectopic budding. The activin signaling pathway is normally active in the WD but was inactivated in the ectopic buds induced by GDNF. Our data suggest that activin A acts as an endogenous inhibitor of UB outgrowth from the WD. GDNF may induce UB outgrowth from the WD by canceling the action of activin A.

Materials and methods

Reagents

Recombinant human activin A, activin B, activin AB, TGF-β1, BMP-2, BMP-4, BMP-7, noggin, chordin, and rat glial-cell-line-derived neurotrophic factor (GDNF), goat anti-βA subunit of activin A antibody, goat anti-activin receptor type IB (ActRIB) antibody, goat anti-activin receptor type IIA (ActRIIA) antibody, goat anti-activin receptor type IIB (ActRIIB) antibody, goat anti-BMP4 antibody, and goat anti-TGF-β (1,2,3) antibody were from R&D systems (Minneapolis, MN). Goat polyclonal antibodies against Smad2/3 or Smad1/5/8 and rabbit anti-β actin antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies against phospho-Smad2/3, phospho-Akt phospho-extracellular signal regulated kinase (ERK), and phospho-p38 were from Cell Signaling Technology (Beverly, MA). Bromodeoxyuridine (BrdU) was from Sigma (St. Louis, MO). FITC-conjugated or Rhodamine-conjugated Dolichos Biflorus (DB) lectin was from Vector Laboratories (Burlingame, CA). Fetal bovine serum (FBS) was from Biowhittaker (Walkersville, MD). DAPI (4′, 6-diamino-2′-phenylindole dihydrochloride) was from Calbiochem (San Diego, CA). DMEM/F12 was purchased from GIBCO-BRL (Grand Island, NY).

Isolation and culture of Wolffian duct

Timed pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN) at day 13 of gestation (day 0 being the day of appearance of the vaginal plug) were used for experiments. The embryos were dissected free of surrounding tissues, and the WD with surrounding mesonephros were isolated under a stereomicroscope. When WDs were cultured in the absence of mesonephros, surrounding mesonephros was removed from the WD with fine forceps, but there were still a small number of mesodermal cells attached on the WD. Isolated tissues were applied to the top of Transwell filters (0.4 μm pore size) (Costar, Cambridge, MA) placed within individual wells of a 12-well tissue culture dish. The isolated tissues were cultured in DMEM/F12 supplemented with 10% FBS at 37°C and 5% CO_2/100% humidity. After the indicated number of days, cultures were fixed with 4% paraformaldehyde (PFA) and used for histological analysis. For the bead experiments, heparin acrylamide beads (Sigma, St. Louis, MO) were incubated in 10 μg/ml of recombinant Follistatin for 1 h on ice and washed briefly in phosphate-buffered saline (PBS) before placing on organ cultures. The care and use of animals described in this study conform to the procedures of the laboratory's Animal Protocol approved by the Animal Subjects Program of the University of California, San Diego.

Quantification of bud formation

For quantification of bud formation, the number of WDs in which ectopic buds was observed regardless of bud number or shape was counted and was expressed as the percentage of total number of WDs used (n = 4–5). Values are the mean ± SE of three to five separate experiments.

DB lectin staining

To visualize the WD and ectopic budding structures specifically, FITC-conjugated or Rhodamine-conjugated Dolichos Biflorus (DB) lectin staining was performed as described previously (Qiao et al., 1999).

Immunohistochemistry

Isolated WDs with mesonephros were cultured on Transwell filter in the presence of the indicated growth factors. After the indicated days, cultures were fixed with 4% PFA and were processed for paraffin-embedded or frozen sections. Immunofluorescent staining was performed as described previously (Maeshima et al., 2002).

Detection of cell proliferation and apoptosis

Cultured WDs were labeled with 100 μM BrdU for 3 h. After extensive washing with PBS, the WDs were fixed with 4% PFA. Cell proliferation was analyzed by BrdU labeling using a cell proliferation kit (Amersham, Piscataway, NJ) (Maeshima et al., 2001b). For identification of nuclei with DNA strand breaks at the cellular level, the terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labeling (TUNEL) staining was performed using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instruction. Quantification of BrdU-positive or TUNEL-positive cells was performed by counting positive nuclei in total cells (DAPI-positive nuclei) from selected fields under fluorescent microscope at ×400 magnification. Values are the mean ± SE (n = 5).

Reverse transcriptase-PCR

Total RNA was isolated with the TRIzol Reagent (Life Technologies/BRL) from WDs or surrounding mesonephros. First-strand cDNA was made from total RNA using Superscript Preamplification System (Life Technologies/BRL) according to the manufacturer's instruction. Contaminated genomic DNA was removed with RNase-free deoxyribonuclease (DNase). Five micrograms of DNase-treated RNA was incubated with 1 μl of oligodT at 70°C for 10 min. Two-microliter 10× PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 1 μl of DTT (0.1 M), 2 μl of dNTP mix (10 mM), and 2 μl of $MgCl_2$ (25 mM) were added to each reaction. After incubation for 5 min at 42°C, 1 μl of reverse transcriptase (RT) was added. Samples were incubated at 42°C for 50 min and then at 70°C for