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Role of mesangial cells and gap junctions in tubuloglomerular feedback

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Background. Tubuloglomerular feedback (TGF) is a process whereby the resistance of the afferent arterioles delivering blood to the glomeruli is regulated by the NaCl concentration of the forming urine in the lumen of the macula densa. Intraglomerular mesangial cells are located between capillaries within the glomerulus, while extraglomerular mesangial cells are located between the macula densa and the afferent arteriole. They are electrically and chemically coupled via gap junctions. The purpose of this study was to investigate the role of mesangial cells and gap junctions in TGF using the isolated, perfused juxtaglomerular apparatus.

Method. Juxtaglomerular apparatuses were dissected from male New Zealand white rabbits and perfused in vitro. The NaCl concentration at the macula densa was changed from 17/2 to 65/50 Na/Cl to initiate a TGF response. Afferent arterioles were perfused at 60 mm Hg throughout the experiment. Changes in luminal diameter caused by increasing the NaCl concentration at the macula densa were taken as the TGF response. TGF was measured before and after disrupting the gap junctions or damaging the mesangial cells in paired experiments.

Results. During the control period, TGF decreased afferent arteriole diameter by 2.9 ± 0.2 μm. After mesangial cells were damaged by perfusing Thy 1-1 antibody and complement into the afferent arteriole, the TGF response was completely eliminated. Separate experiments showed no statistically significant change in TGF response with time, or when antibody and complement were perfused into the macula densa lumen. The presence of Thy 1-1 antibody and complement in the afferent arteriole perfusate did not alter the ability of norepinephrine to constrict or acetylcholine to dilate the afferent arteriole. To investigate the role of gap junctions in TGF, we used heptanol to disrupt them. During the control period, TGF decreased afferent arteriole diameter by 2.9 ± 0.4 μm. After perfusing heptanol into the lumen of the afferent arteriole, the TGF response was completely eliminated. When heptanol was added to the bath, it had no significant effect on TGF response.

Discussion. The data show that after mesangial cells were selectively damaged, the constriction of the afferent arteriole induced by increasing the NaCl concentration at the macula densa was eliminated. However, such treatment had no effect when Thy 1-1 was perfused into the macula densa lumen, and did not alter the response of the afferent arteriole to norepinephrine or acetylcholine. Disruption of the gap junctions also eliminated the TGF response. These data indicate that the mesangial cells play a key role in mediating the TGF response, and that gap junctions among mesangial cells and between mesangial cells and vascular smooth muscle cells communicate the TGF signal to the afferent arteriole.

Key words: cell signaling, macula densa, TGF response, afferent arteriole, vascular smooth muscle cells, immune response.

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Tubuloglomerular feedback (TGF) is a process whereby the resistance of the afferent arterioles delivering blood to the glomeruli is regulated by the NaCl concentration of the forming urine in the lumen of the macula densa. As the NaCl concentration increases, the resistance of the afferent arteriole increases in a process called TGF. The macula densa is a small plaque of cells that forms part of the nephron distal to the loop of Henle and is thought to be the sensor of the luminal NaCl concentration for TGF [1, 2]. Thus, the macula densa is considered the initiation site of TGF. The final step in the process is constriction of the afferent arteriole [3–5].

Glomerular capillaries have a small juxtamesangial portion that is not underlain by a basement membrane, leaving an area of direct contact between mesangial cells and endothelium. Therefore, the capillary-mesangium interface consists of a fenestrated endothelium, where water, small solutes and uncharged macromolecules in the blood freely pass through the endothelium to the mesangial cells [6]. Although the macula densa does not contact the vessel directly, its basolateral aspect is closely associated with extraglomerular mesangial cells, which in turn are in contact with intraglomerular mesangial cells, and both the intra- and extraglomerular mesangial cells contact the vascular smooth muscle and endothelial cells of the afferent arteriole [7]. There are numerous gap junctions between the extra- and intraglomerular mesangial cells as well as between the extraglomerular mesangial cells and the vascular smooth muscle cells of the afferent arteriole. Consequently, these cells are thought to act as a syncytium [8–12].

The nature of the signal emitted by the macula densa that initiates TGF is unknown. However, given the anatomy of the juxtaglomerular apparatus, which includes the macula densa, extraglomerular mesangial cells, glomerulus and afferent arteriole, it has been proposed that mesangial cells may mediate TGF due to their central location between the macula densa and afferent arteriole. Several investigators have shown that in vivo elimination of these cells diminishes the feedback response [13, 14], but interpretation of these data is not straightforward because the technique used to destroy the mesangial cells also caused an inflammatory response. Furthermore, little is known about the role of the gap junctions between the various cells in TGF. The purpose of our study was to investigate the role of the mesangial cells and the gap junctions between mesangial cells and vascular smooth muscle cells in TGF using the isolated, perfused juxtaglomerular apparatus. In this preparation there is no immune response when antigen/complement is used to disrupt cells.

METHODS

Tubuloglomerular feedback

Tubuloglomerular feedback was measured as described previously [15, 16]. Briefly, young male New Zealand white rabbits (Covance, Denver, PA, USA) were given tap water ad libitum and fed standard rabbit chow. The rabbits were anesthetized with ketamine (50 mg/kg, IM) and given an IV injection of heparin (500 U). The kidneys were removed and sliced along the corticomedullary axis. Slices were placed in ice-cold minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) and dissected under a stereomicroscope (Olympus SZH; Tokyo, Japan). From each rabbit, a single superficial afferent arteriole and its intact glomerulus were microdissected together with adherent tubular segments consisting of portions of the thick ascending limb of the loop of Henle, macula densa, and early distal tubule. The sample was transferred to a temperature-regulated chamber mounted on an inverted microscope (Olympus IMT-2) with Hoffmann modulation. Both the afferent arteriole and the end of either the distal tubule or thick ascending limb were cannulated with an array of glass pipettes. Intraluminal pressure of the afferent arteriole was measured by Landis' technique, using a fine pipette introduced into the lumen through the perfusion pipette. The afferent arteriole was perfused with oxygenated MEM (95% O_2; 5% CO_2) containing 5% BSA, and intraluminal pressure was maintained at 60 mm Hg throughout the experiment. Two solutions were used to perfuse the macula densa. The first contained (in mmol/L) 15 $NaCO_3$; 0.96 NaH_2PO_4; 0.24 Na_2HPO_4; 5 $KHCO_3$; 1.2 $MgSO_4$; 1 $CaCl_2$; 5.5 glucose; and 1 Na acetate (oxygenated to pH 7.4) at a rate of 10 nL/min; thus the final concentrations were 17.4 mmol/L Na and 2 mmol/L Cl. The other solution had a similar composition except that 48 mmol/L NaCl was added; thus the final concentrations were 65.4 mmol/L Na and 50 mmol/L Cl. The bath consisted of 100 μL MEM containing 0.15% BSA and was exchanged continuously at a rate of 1 mL/min. Microdissection and cannulation were completed within 90 minutes at 8°C, after which the bath was gradually warmed to 37°C for the rest of the experiment. Once the temperature was stable, a 30-minute equilibration period was allowed before taking any measurements. Images were displayed at magnifications up to ×1980 and recorded with a Sony video system consisting of a camera (DXC-755), monitor (PVM1942) and video recorder (EDV-9500). The diameter of the distal afferent arteriole was measured with an image analysis system (Universal Imaging, West Chester, PA, USA) at the site of maximal response.

Experimental protocols

Protocol 1. Effect of Thy 1-1 antibody plus complement in the afferent arteriole lumen on TGF. Once the preparation was equilibrated, TGF was measured by perfusing the macula densa with low NaCl solution, waiting 10 minutes before measuring afferent arteriole diameter, then switching the macula densa perfusate to high NaCl solution and measuring diameter 10 minutes later. Then the macula densa perfusate was switched back to low NaCl solution and the afferent arteriole was perfused with medium containing Thy 1-1 antibody (1:1000; Sigma) and complement (40 CH50 U/mL; Sigma) for one hour. Following a 10-minute washout period, the NaCl concentration at the macula densa was changed as described for the control period to measure TGF.

To show that Thy 1-1 antibody and complement treatment damages mesangial cells, glomeruli were examined by transmission electron microscopy. Glomeruli were fixed in 100 mmol/L sodium cacodylate buffer containing 3.0% glutaraldehyde. Then they were post-fixed in 1.0% OsO_4, dehydrated in ethanol, and embedded in araldite resin. Thin sections were cut, stained with lead citrate and uranyl acetate, and viewed with a Philips 201 transmission electron microscope. Mesangial cells in antibody/complement-treated glomeruli had disrupted plasma membranes and loss of mitochondrial cristae (Fig. 1).

Protocol 2. Effect of Thy 1-1 antibody plus complement in the macula densa lumen on TGF. To determine whether Thy 1-1 antibody damages the macular densa cells directly, it was added to the tubular lumen. After the control period, the macula densa perfusate was switched back to low NaCl solution containing Thy 1-1 antibody (1:1000; Sigma) and complement (40 CH50 U/mL; Sigma) for one hour as in Protocol 1. Following a 10-minute washout period, the NaCl concentration at the

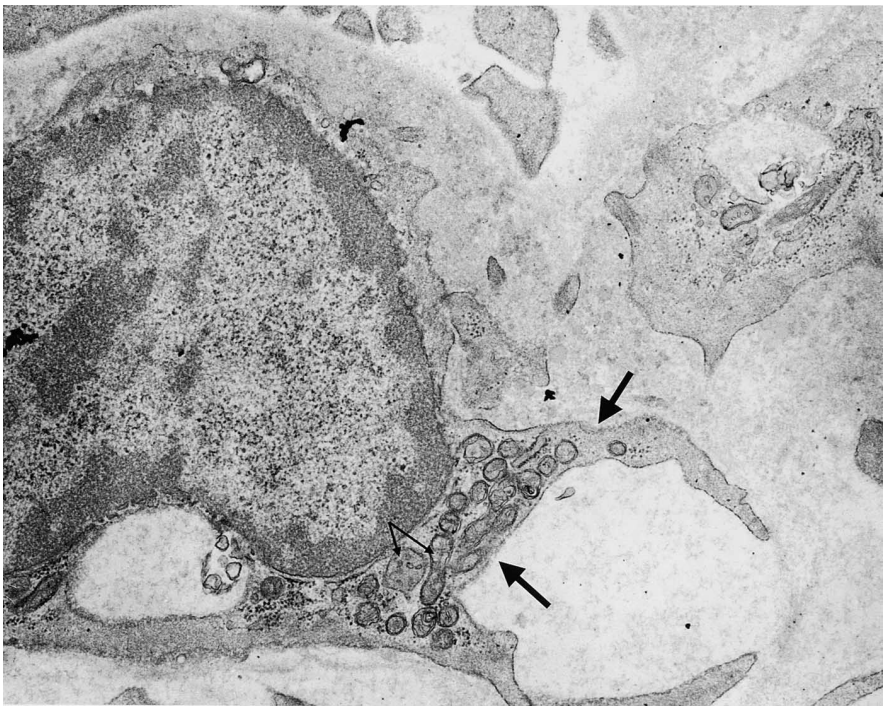

Fig. 1. Electron micrograph of a representative mesangial cell from a glomerulus treated with Thy 1-1 antibody and complement. Note the treatment-induced mesangial cell damage consisting of marked loss of mitochondrial cristae (small arrows) and disruption of the plasma membrane (large arrows).

macula densa was changed as described for the control period to measure TGF.

Protocol 3. Effect of Thy 1-1 antibody plus complement on afferent arteriole reactivity. In separate experiments, to measure afferent arteriole reactivity, vessels were treated with Thy 1-1 antibody plus complement as described above. Afferent arterioles were preconstricted by adding norepinephrine to the bath, and 10 minutes later acetylcholine was added to the lumen at 10^{-6} and 10^{-5} mol/L for 10 minutes at each dose.

Protocol 4. Effect of factor VIII-related antigen antibody plus complement on TGF. To study Thy 1-1 antibody specificity and the role of endothelial nitric oxide synthase (eNOS) in TGF, we used factor VIII-related antigen antibody plus complement, which damages the afferent arteriole endothelium. After the control period, the macula densa perfusate was switched back to low NaCl solution and the afferent arteriole was perfused with medium containing factor VIII-related antigen antibody (1:1000; Incstar, Stillwater, MN, USA) and complement (40 CH50 U/mL; Sigma) for 10 minutes. Following a 20-minute washout period, the NaCl concentration at the macula densa was changed as described for the control period to measure TGF.

Protocol 5. Effect of heptanol in the afferent arteriole lumen on TGF. Gap junctions reportedly exist within the juxtaglomerular apparatus. To study their role in TGF, heptanol was used to disrupt them. After the control period, the macula densa perfusate was switched back to low NaCl solution and 1 mmol/L heptanol was added to the afferent arteriole perfusion solution. After 10 minutes, the NaCl concentration at the macula densa was changed as described for the control period to measure TGF.

Protocol 6. Effect of heptanol in the bath on TGF. This was similar to Protocol 5 except that 1 mmol/L heptanol was added to the bath. After 10 minutes, the NaCl concentration at the macula densa was changed as described for the control period to measure TGF.

Statistics

Values are expressed as mean ± SEM. A paired *t* test was used to examine whether the diameter at a given concentration was different from control. Analysis of variance (ANOVA) was used to examine whether dose-response curves differed between groups, and a two-sample *t* test was used to examine whether the changes in diameter at a given concentration differed between groups. $P < 0.025$ was considered significant using Bonferroni's correction for multiple comparisons.

RESULTS

First, the effect of disrupting mesangial cells with Thy 1-1 antibody and complement on TGF was examined (Fig. 2). During the control period, TGF decreased afferent arteriole diameter by 2.9 ± 0.2 μm when the solution perfusing the macula densa was switched from 17 mmol/L Na/2 mmol/L Cl to 65 mmol/L Na/50 mmol/L Cl. When Thy 1-1 antibody and complement were added

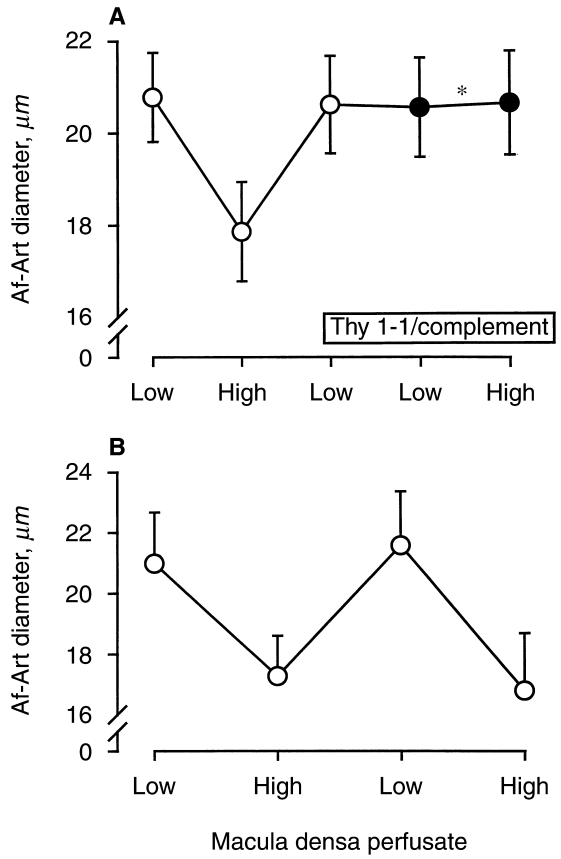

Fig. 2. (*A*) Effect of disrupting mesangial cells on tubuloglomerular feedback (TGF). Mesangial cells were disrupted by perfusing the afferent arteriole (Af-Art) with Thy1-1 antibody and complement. *$P <$ 0.001, control TGF response vs. antibody treatment ($N = 6$). Symbols are: (○) control period; (●) after adding Thy 1-1 antibody plus complement. When the NaCl concentration was increased from low to high, the Af-Art diameter decreased by 2.9 μm. Treatment with Thy1-1 antibody and complement had no effect on basal diameter but completely blocked the constriction induced by high NaCl at the macula densa. (*B*) Time-control experiments demonstrated that this constriction was reproducible.

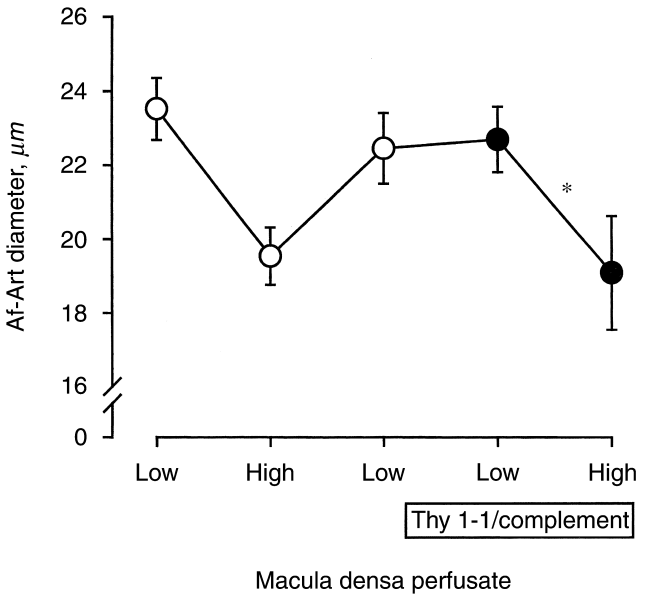

Fig. 3. Effect of adding Thy 1-1 antibody and complement to the perfusate of the macula densa. *$P = 0.76$, control TGF response vs. antibody treatment ($N = 6$). Symbols are: (○) control period; (●) after adding Thy 1-1 antibody plus complement. Perfusion of the macula densa with Thy 1-1 antibody and complement had no effect on TGF.

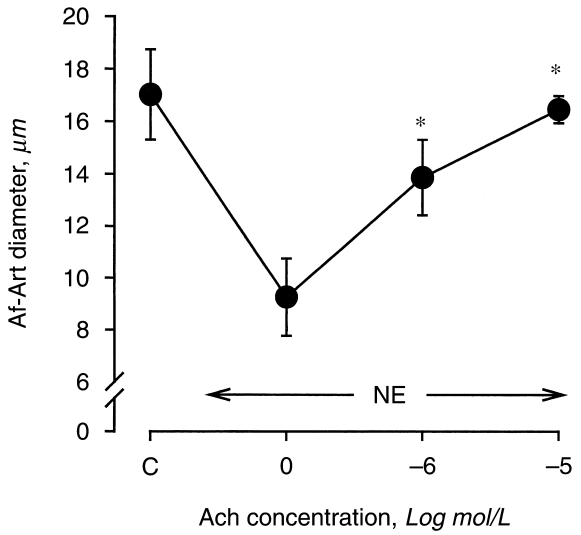

Fig. 4. Response of preconstricted afferent arteriole (Af-Art) to acetylcholine (Ach) after treatment with Thy 1-1 antibody plus complement. *$P < 0.005$, Ach vs. NE alone ($N = 6$). Perfusion of the afferent arteriole with Thy 1-1 antibody plus complement did not alter the afferent arteriolar reactivity.

to the afferent arteriole perfusate, the TGF response was completely eliminated (−0.2 ± 0.2 μm). The paired difference between control TGF and TGF response to antibody treatment was 3.1 ± 0.3 μm ($P < 0.001$). In separate experiments, no significant change in the TGF response was found with time ($N = 6$).

To demonstrate that the effect of Thy 1-1 antibody is selective for mesangial cells, its effect in the lumen of the macula densa was examined. During the control period, in these experiments the TGF response decreased afferent arteriole diameter by 3.8 ± 0.4 μm. After Thy 1-1 antibody and complement were perfused into the macula densa, the TGF response was 3.6 ± 0.9 μm. The paired difference in the TGF response between the control period and after the macula densa had been treated with antibody was –0.1 ± 0.9 μm ($P = 0.76$; Fig. 3).

To show that Thy 1-1 antibody and complement do not alter vascular smooth muscle or endothelial cell function, we used them to treat the afferent arterioles and investigate the ability of norepinephrine and acetylcholine to contract and dilate the vessels, respectively (Fig. 4). In afferent arterioles treated with Thy 1-1 antibody and complement, when norepinephrine was added to the

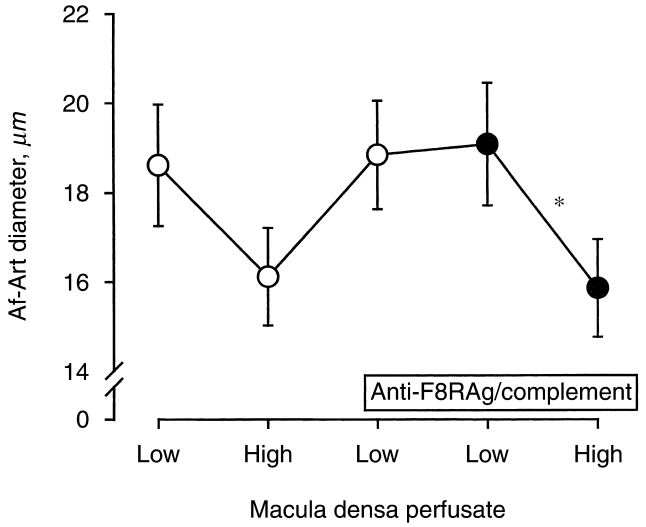

Fig. 5. Effect of disrupting endothelial cells on tubuloglomerular feedback (TGF). Endothelial cells were disrupted by perfusing the afferent arteriole with factor VIII-related antigen antibody (AntiF8RAg) and complement. *P = 0.16, control TGF response vs. antibody treatment (N = 6). Symbols are: (○) control period; (●) after adding factor VIII-related antigen antibody plus complement. Damaging endothelial cells did not affect TGF.

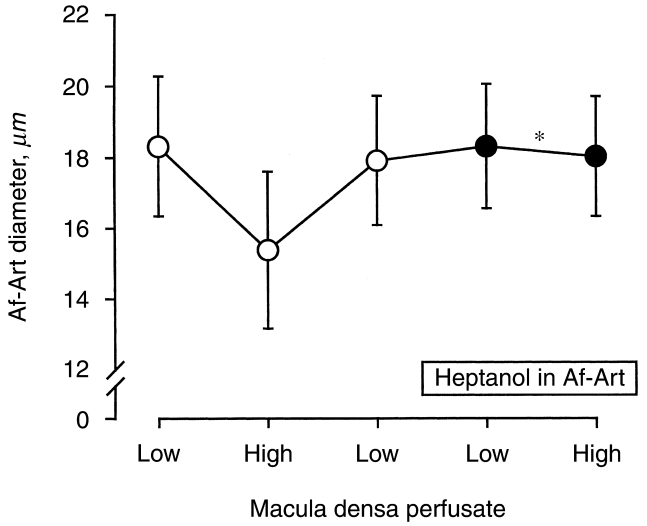

Fig. 6. Effect of disrupting gap junctions with heptanol on tubuloglomerular feedback (TGF). *P = 0.002, control TGF response vs. heptanol treatment (N = 6). Symbols are: (○) control period; (●) heptanol 1 mmol/L added to the afferent arteriole lumen. Disrupting gap junctions within the juxtaglomerular apparatus inhibited TGF.

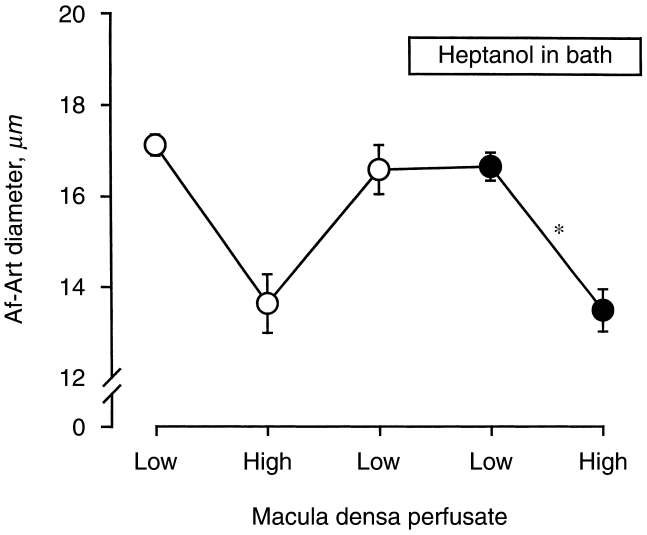

Fig. 7. Effect of disrupting gap junctions with heptanol on tubuloglomerular feedback (TGF) when it was added to the bath. *P = 0.61, control TGF response vs. heptanol treatment (N = 6). Symbols are: (○) control period; (●) heptanol 1 mmol/L added to the bath. Adding heptanol to the bath had no effect on TGF.

bath, the diameter decreased from 17.0 ± 1.7 μm to 9.3 ± 1.5 μm. Acetylcholine at 1 and 10 μmol/L dilated arterioles to 13.9 ± 1.5 μm and 16.6 ± 0.5 μm, respectively.

To investigate the role of the afferent arteriole endothelium in TGF, we disrupted it by treatment with an antibody against factor VIII-related antigen and complement (Fig. 5) [17]. During the control period, TGF decreased afferent arteriole diameter by 2.5 ± 0.6 μm. After the afferent arteriole had been treated with an antibody against factor VIII-related antigen and complement, the TGF response induced by increasing the NaCl concentration at the macula densa was 3.3 ± 0.4 μm. The paired difference in the TGF response between the control period and after the afferent arteriole had been treated with factor VIII-related antigen antibody and complement was 0.8 ± 0.4 μm (P = 0.16).

To investigate the role of gap junctions in TGF, heptanol was used to disrupt them [18]. During the control period, TGF decreased afferent arteriole diameter by 2.9 ± 0.4 μm. After perfusing 1 mmol/L heptanol into the afferent arteriole lumen, the TGF response induced by increasing the NaCl concentration at the macula densa was 0.3 ± 0.3 μm. The paired difference in the TGF response between the control period and heptanol treatment was 2.7 ± 0.4 μm (P = 0.002; Fig. 6). In contrast, when 1 mmol/L heptanol was added to the bath, it had no statistically significant effect on the TGF response induced by increasing the NaCl concentration at the macula densa (control period, 3.5 ± 0.5 μm; heptanol treatment, 3.2 ± 0.4 μm; paired difference −0.3 ± 0.5 μm; Fig. 7).

DISCUSSION

Our data show that after disrupting mesangial cells by adding Thy 1-1 antibody and complement to the afferent arteriole lumen, the vasoconstriction of the afferent arteriole induced by TGF is eliminated. Such a treatment had

no effect when it was perfused into the tubular segment containing the macula densa, nor did it alter the response of the afferent arteriole to norepinephrine or acetylcholine. Additionally, disrupting endothelial cells with a similar treatment using an antibody selective for factor VIII-related antigen, had no effect on the vasoconstriction induced by TGF. Given that maneuvers intended to disrupt the endothelial cells did not alter TGF, and that vascular smooth muscle cells in the afferent arteriole were unaffected by Thy 1-1 antibody and complement, we conclude that disruption of mesangial cells in an in vitro preparation significantly blunts TGF.

The role of mesangial cells in TGF and in regulation of the glomerular filtration rate (GFR) is still controversial. Many investigators have argued that mesangial cells serve only as scaffolding for the endothelial and epithelial cells of the glomerulus and are not involved in GFR [19, 20]. Others have provided evidence that mesangial cells play an important role in regulating GFR by changing the surface area available for filtration [7, 21, 22]. More recently, Yamamoto et al provided in vivo evidence that mesangial cells are important for maintaining GFR and probably TGF [14]. However, some caution must be used when interpreting these findings due to the inflammation associated with disruption of mesangial cells in vivo using Thy 1-1 antibody and complement. By performing our studies in vitro and directly measuring the effect of changing the NaCl concentration at the macula densa on afferent arteriole diameter, we were able to directly address the question of whether intact mesangial cells are necessary for TGF. However, the limitations of such experiments must be recognized.

To assure that the effect of perfusing Thy 1-1 antibody and complement into the lumen of the afferent arteriole was due to destruction of mesangial cells, two separate experiments were performed. First, Thy 1-1 antibody and complement were perfused into the macula densa lumen. Additionally, an antibody against factor VIII-related antigen and complement were perfused into the lumen of the afferent arteriole. Neither treatment had any effect on TGF response. These data suggest that perfusing Thy 1-1 antibody and complement into the lumen of the afferent arteriole diminishes the TGF response by killing mesangial cells.

In the juxtaglomerular apparatus there are numerous gap junctions between many cell types [11, 12, 23]. There are gap junctions between intra- and extraglomerular mesangial cells, between mesangial cells and vascular smooth muscle cells, and among vascular smooth muscle cells [8, 12, 23]. The role of these gap junctions in TGF is not well defined. This is primarily due to the difficulty of selectively disrupting gap junctions in vivo and the technical difficulty associated with measuring TGF in vitro. It is well known that gap junctions are blocked by heptanol [24, 25] and can be activated or recruited by tetraethylammonium [26, 27]. In cultured vascular smooth muscle cells, heptanol (0.01 and 1 mmol/L) inhibited dye transfer in a concentration-dependent manner in the presence of tetraethylammonium [18]. Our study demonstrates that intact gap junctions are necessary for increased NaCl concentration at the macula densa to elicit a TGF response. While we could not pinpoint the location of the gap junctions required for TGF, we did note that adding heptanol to the bath had no effect on TGF. In contrast, adding it to the perfusate of the afferent arteriole did blunt TGF. Thus gap junctions either among mesangial cells or between mesangial cells and vascular smooth muscle cells appear to be necessary for the TGF response to be transmitted from the macula densa to the afferent arteriole. Gap junctions among vascular smooth muscle cells do not appear to be important for TGF. The gap junctions among vascular smooth muscle cells would be exposed to heptanol in the bath but not in the afferent arteriole lumen. Heptanol in the bath does not reach the mesangial cells, since filtration proceeds from inside to outside due to differences in pressure and Bowman's capsule serves as a barrier; conversely, heptanol in the lumen of the arteriole reaches the mesangial cells through the capillary-mesangium interface. These data also imply that mesangial cells may transduce the signal released from the macula densa that initiates a TGF response, and that the mesangial cells then convey that signal to the vascular smooth muscle cells of the afferent arteriole via either an electrical or chemical pathway.

In summary, we have shown that intact mesangial cells and gap junctions are necessary for TGF. Our data imply that the signal released from the macula densa in response to an increase in NaCl concentration in the tubular lumen is first transduced by the mesangial cells before it is passed to the vascular smooth muscle cells of the afferent arteriole.

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