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Research Paper

Incubation of Metanephroi with Vitamin D_3 Increases Numbers of Glomeruli

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KEY WORDS

kidney, organogenesis, transplantation

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ABSTRACT

To characterize actions of vitamin D_3 on metanephroi transplanted from rat embryos to adult recipients, we incubated metanephroi with or without 0.01, 0.1 or 1 ug/ml vitamin D3, 25-hydroxyvitamin D_3 [25(OH)D_3] or 1, 25-hydroxyvitamin D_3 [1,25$(OH)_2D_3$] prior to implantation. The number of glomeruli in developed metanephroi three weeks post-transplantation that had been incubated with 1.0 ug/ml vitamin D_3 was increased relative to the number in metanephroi that were not incubated with vitamin D_3 (control), an effect that was not recapitulated by administration of vitamin D_3 directly to hosts at the time of transplantation. Incubation of metanephroi with 1.0 ug/ml vitamin D_3 also enhanced inulin clearances of metanephroi measured at 12 weeks post-transplantation. The hydroxylated derivative of vitamin D_3, 25(OH)D_3, increased glomerulus number when applied at 0.01ug/ml but not at higher concentrations, while the twice-hydroxylated derivative 1,25$(OH)_2D_3$, failed to increase glomerulus number at any concentration tested. We conclude that incubation with vitamin D_3 prior to implantation enhances inulin clearance possibly by increasing the number of glomeruli that develop post-transplantation.

Our findings suggest the vitamin D_3 effect is mediated locally.

INTRODUCTION

We have shown that metanephroi from rat embryos transplanted into the omentum of adult rat hosts undergo growth, differentiation, and vascularization, clear inulin from the host's circulation after anastomosis between the ureter of the transplant and host[1] and can support life in otherwise anephric recipients.[2] Successful xenotransplantations of metanephroi have also been performed.[3-5] Xenotransplantation (pig into mouse) was optimal under conditions such that renal anlagen were incubated prior to implantation in a solution that contained a number of growth factors and other growth promoting agents including vitamin D_3.[4]

Vitamin D_3, an endogenous steroid synthesized in the skin, is biologically inactive. Formation of its major biologically active product, 1, 25-hydroxyvitamin D_3 [1,25$(OH)_2D_3$], results from hydroxylations at position C-25 primarily in the liver and C1α primarily in the kidney.[6,7] Therefore, an action of vitamin D_3 per se on transplanted metanephroi would be unexpected.

To shed light on whether incubation of metanephroi with vitamin D_3 alone prior to implantation affects subsequent differentiation and function, we incubated metanephroi from rat embryos with or without vitamin D_3, 25(OH)D_3, or 1,25$(OH)_2D_3$ in the absence of other growth factors. Here we show that incubation with 1.0 ug/ml vitamin D_3 prior to implantation increases the number of glomeruli measured at three weeks post-transplantation, and enhances inulin clearances measured in metanephroi 12 weeks post-transplantation. The action of vitamin D_3 in vitro to increase numbers of glomeruli is not recapitulated by systemic administration of vitamin D_3 to hosts.

METHODS

Transplantation of metanephroi, measurement of inulin clearance and histology. Metanephroi were surgically dissected from embryonic day (E) 15 Sprague-Dawley rat embryos or, where indicated Lewis rat embryos, under a dissecting microscope using previously described techniques.[1] Immediately after dissection metanephroi were placed in 1 ml of a 50:50 mixture of Ham's F12:Dulbecco's modified Eagles medium at 4°C containing 25 nM prostaglandin E1, and iron-saturated transferrin (5 ug/ml) (HF12:DMEM) for 45 minutes.[1-4] When indicated, the following additions were made to the HF12:DMEM (incubation solution): none (control); 0.01, 0.1, or 1.0

ug/ml vitamin D_3 (Sigma Chemicals, St. Louis MO), $25(OH)D_3$, or $1,25(OH)_2D_3$ (provided by Dr. Milan Uskokovic, Hoffman-La Roche Nutley NJ); 10^{-7} M recombinant human insulin-like growth factor I (IGF I) (Genentech Inc. S. San Francisco CA); or 10^{-6} M retinoic acid (RA) (Sigma Chemicals). Metanephroi were removed from the incubation solution, and implanted in the omentum of anaesthetized six week old female (host) Sprague Dawley or Lewis rats. During the same surgery, host rats had one kidney removed.

In some hosts, three weeks following transplantation, end-to-end ureter-oureterostomy was performed using microvascular technique (interrupted 10-0 suture) between the ureter of a metanephros implanted in the omentum and the ureter of the kidney that had been removed. Nine weeks later all remaining native renal tissue (the contralateral kidney) was removed from host rats, following which inulin clearances were measured on conscious rats after placement of an indwelling bladder catheter and intravenous line exactly as in previous studies.[1] Baseline measurements for inulin were performed on urine and blood samples obtained prior to beginning the inulin infusions. These "background" values were subtracted from measurements performed after beginning the inulin infusion. Infusion was begun only following removal of all remaining native renal tissue and drainage of all urine remaining in the bladder (10–20 ul). Rats received no immunosuppression.[1]

Determination of glomerulus number. Numbers of glomeruli per whole metanephros were determined using a technique described by Nathanson et al.[8] with modifications. Briefly, whole metanephroi were incubated in 6N hydrochloric acid for 45–60 minutes at 37°C, rinsed with tap water, and stored overnight at 4°C in a volumetric flask. The kidneys were mechanically dissociated and the tubules and glomeruli suspended in water. Three 0.5 ml aliquots were taken, the glomeruli were counted under a dissecting microscope.

Multiple comparisons were made using Student- Newman-Keuls Test.

RESULTS

Initial experiments were performed to determine whether incubation of metanephroi with vitamin D_3 prior to implantation affects numbers of nephrons formed post-transplantation. To this end, we measured numbers of glomeruli in metanephroi incubated prior to implantation in DMEM: HF12 (control media) or 1.0 ug/ml vitamin D_3, RA, known to increase the number of nephrons in developing kidneys several-fold[9] as a positive control rIGF I, an agent that has been shown to be a more modest (~20%) stimulator of nephron numbers[10] as a negative control.

The number of glomeruli in metanephroi that had been incubated in control media was 4140 ± 596 or about 13% of the number present in an adult rat kidney.[11] Incubation with RA enhanced significantly the number of glomeruli to 9010 ± 1207, or to about 30% of normal. Incubation with vitamin D_3 had a comparable effect (9620 ± 1139). In contrast, incubation with IGF I, did not significantly increase glomerulus numbers (Table 1). Numbers of glomeruli expressed per mg metanephros weight are also significantly higher than control in metanephroi incubated with vitamin D_3 or RA (Table 1).

To exclude the possibility that incubation of metanephroi with vitamin D_3 prior to implantation increases the number of glomeruli post-transplantation by virtue of administration of vitamin D_3 to hosts per se (via implantation of vitamin D_3-incubated metanephroi), 1 ug of vitamin D_3 was administered intravenously in 1 ml of sterile saline to Sprague Dawley rat hosts concomitant with transplantation of DMEM:HF12 (control media)-incubated metanephroi. Alternatively, 1 ml of sterile saline was injected. There was no difference between nephron numbers in metanephroi implanted in vitamin D_3-treated rats (5725 ± 803) (n = 10) or saline-treated rats (4284 ± 396) (n = 12).

We next incubated metanephroi prior to implantation in DMEM:HF12 (control media), or HF12:DMEM containing varying concentrations of vitamin D_3 (0.01, 0.10, or 1.0 ug/ml) and counted glomeruli three weeks post-transplantation. Numbers of glomeruli were increased significantly compared to control in metanephroi that had been incubated in DMEM:HF12 containing 1.0 ug/ml vitamin D_3, but not the two lower concentrations (Fig. 1).

Table 1 **Numbers of glomeruli in metanephroi**

Incubation	Control	Vitamin D_3	Vitamin A	IGF I
Number of experiments	(6)	(6)	(6)	(6)
Metanephros weight (mg)	62 ± 3.7	71 ± 5.9	52 ± 1.8	45 ± 2.0
Glomeruli	4140 ± 569	9620 ± 1139*	9010 ±1207*	5320 ± 964
Glomeruli per mg metenephros weight	66 ± 8.0	140 ± 23**	174 ± 22**	114 ± 18

Transplantations were carried out from Lewis rat embryos to adult Lewis rats. Data are presented as mean ± SEM different than control. **$p<0.05$; *$p<0.01$.

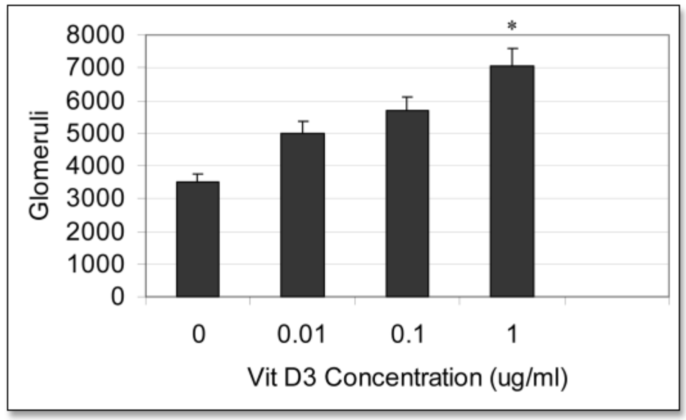

Figure 1. Numbers of glomeruli in metanephroi transplanted from Sprague Dawley rat embryos to adult Sprague Dawley rats. Metanephroi were incubated in DMEM:HF12 without vitamin D_3 (0) (n = 28) or containing 0.01 ug/ml (n = 8); 0.1 ug/ml (n = 9) and 1.0 ug/ml (n = 28) Vitamin D_3. Data are mean ± SEM. * Greater than control p < 0.005.

Table 2 **Numbers of glomeruli in metanephroi**

Incubation	n	Glomeruli
Control	12	3673 ± 353
$25(OH)D_3$		
0.01ug/ml	9	*7384 ± 610
0.1 ug/ml	8	6092 ± 713
1.0 ug/ml	8	3723 ± 769
$1,25(OH)_2D_3$		
0.01ug/ml	12	4464 ± 504
0.1 ug/ml	5	6490 ± 945
1.0 ug/ml	4	5067 ± 363

Transplantations were carried out from Sprague Dawley rat embryos to adult Sprague Dawley rats. Data are presented as mean ± SEM. *Different than control $p<0.05$.

Table 3 **Weights, urine volumes and inulin clearances of metanephroi**

Incubation	Control	Vitamin D_3
Number	(4)	(3)
Met Weight (mg)	65 ± 18	79 ± 8.0
Host Weight (g)	245 ± 8.0	270 ± 8.5
Urine vol. (ul/hour)	51 ± 7.1	128 ± 9.3*
Inulin Clearance (ul/min/100mg)	0.43 ± 0.06	1.1 ± 0.06**

Transplantations were carried out from Sprague Dawley rat embryos to adult Sprague Dawley rats. Data are presented as mean ± SEM. *Vitamin D_3>Control $p<0.05$; **Vitamin D_3>Control $p<0.01$.

To compare actions of vitamin D_3 on glomerulus numbers with actions of hydroxylated metabolities of vitamin D_3, we counted numbers of glomeruli three weeks post-transplantation in metanephroi that had been incubated in DMEM:HF12 containing 0.01, 0.10 or 1.0 ug/ml of 25(OH)D_3 or 1,25$(OH)_2D_3$ prior to implantation. The number of glomeruli was increased significantly relative to control only after incubation in 0.01ug/ml 25(OH)D_3 (Table 2).

To determine whether incubation with vitamin D_3 affects function in transplanted metanephroi, urine volumes and inulin clearances were measured at 12 weeks post-transplantation, in metanephroi that had been transplanted following incubation with vehicle or vitamin D_3. Incubation with vitamin D_3 increased both parameters (Table 3).

DISCUSSION

The kidney is a target organ for active forms of vitamin D. The pattern of intracellular 1,25$(OH)_2D_3$ vitamin D receptor (VDR) expression during renal ontogenesis has been defined in the rat. Starting at E15, epitopes for the VDR are found in cells of branching ureteric buds and in the surrounding mesenchyme, and at later stages, in glomerular parietal and visceral epithelial cells.[12] The VDR cannot be detected in metanephroi from E13 rats. However, beginning after 3 days in organ culture, the pattern of expression is identical to that detected in vivo.[12]

Presently we have shown that incubation of metanephroi with vitamin D_3 prior to implantation increases the number of glomeruli three weeks post-transplantation and enhances inulin clearance 12 weeks post-transplantation. In developed kidneys numbers of glomeruli reflect numbers of nephrons since each nephron has a single glomerulus. However, this equation cannot necessarily be made in developing metanephroi. Therefore, it is possible, but not proven that the enhanced inulin clearance reflects larger numbers of nephrons.

It is of interest that incubation of metanephroi with 0.01 ug/ml 25(OH)D_3, but not higher concentrations increases the number of glomeruli. Possibly, the biological activity of 25(OH)D_3 in vitro is explained by its 1α hydroxylation in metanephroi[13,14] to form 1,25$(OH)_2D_3$. However, this is unlikely because 1,25$(OH)_2D_3$ does not recapitulate the 25(OH)D_3 effect.

The mechanisms for these biological actions of vitamin D_3 are not defined in our studies, and the identity of the vitamin D_3 metabolite or metabolites that are biologically active is not delineated. However, since systemic administration of vitamin D_3 at the time of transplantation does not recapitulate the effect of incubation prior to implantation, our findings suggest the effect is mediated locally, and provide a rationale for the use of vitamin D_3 in media for incubation of metanephroi prior to implantation into hosts.

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