Microvascular Research 59, 190–194 (2000)
doi:10.1006/mvre.1999.2214, available online at http://www.idealibrary.com on IDEAL®

BRIEF COMMUNICATION

Modulation of Renal Blood Vessel Formation by Glial Cell Line-Derived Neurotrophic Factor

Sabine Kloth,* and Clemens Suter-Crazzolara†,[1]

*Institute of Anatomy, University of Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany; and †Department of Neuroanatomy, University of Heidelberg, Im Neuenheimer Feld 307, D-69120 Heidelberg, Germany

Received September 28, 1999

Glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-β (TGF-β) superfamily (Roberts and Sporn, 1990). GDNF is important for kidney development. First, the kidney of newborn rodents contains high amounts of GDNF mRNA (Suter-Crazzolara and Unsicker, 1994). GDNF is expressed by the metanephrogenic mesenchyme (Hellmich *et al.*, 1996; Suvanto *et al.*, 1996). GDNF $-/-$ mice die shortly after birth, primarily due to dysgenesis of the kidney (Moore *et al.*, 1996; Pichel *et al.*, 1996; Sanchez *et al.*, 1996). The GDNF receptor Ret can be detected on the epithelium of the uretric bud, while the high affinity receptor GFRα-1 is expressed also in the mesenchyme (reviewed by Sariola and Sainio, 1996). Mice with a disrupted Ret gene reveal a strikingly similar phenotype to GDNF $-/-$ animals (Schuchardt *et al.*, 1996).

The proper function of the kidney depends on the coordinated development of renal vessels, nephrons, and collecting ducts. Several factors such as vascular endothelial growth factor (VEGF; Kloth *et al.*, 1995) or retinoic acid (Kloth *et al.*, 1998) are known to modulate renal vessel growth and differentiation. Here, we have employed an organotypic tissue culture model to investigate the mechanisms by which GDNF may act during renal vessel development.

[1] Present address: DKFZ, Department of Molecular Biophysics H0200, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany.

MATERIALS AND METHODS

Tissue culture. The nephrogenic zone of the kidney was prepared from neonatal rabbits. The explants were cultured for 13 days under continuous medium flow (flow rate: 1 ml/h) in a perfusion culture container (Minuth *et al.*, 1992; Minucell and Minutissue, Bad Abbach, Germany). As basal culture medium IMDM (Gibco BRL-Life Technologies, Eggenstein, Germany) supplemented with 0.1% bovine serum albumin (BSA, Biomol, Hamburg, Germany) was used.

To stimulate renal development, basic culture medium was supplemented with hormones or differentiation factors. Recombinant human GDNF (Prepro Tech Inc., UK) and rat GDNF (Suter-Crazzolara and Unsicker 1995) were applied at a concentration of 5 ng/ml for 13 days of perfusion culture. In another set of experiments explants were stimulated for 1 and 2 days with different hormones. Aldosterone (1×10^{-7}

0026-2862/00 $35.00

M, Sigma, Deisenhofen, Germany) and 1.25-dihydroxyvitamin D_3 (1×10^{-9} M, Sigma) were applied in combination during the whole culture period. Retinoic acid (3.3×10^{-7} M, Sigma, dissolved in ethanol) was applied for 3 h prior to 1 and 2 days of perfusion culture. Control experiments were performed with basal medium alone. All culture experiments were carried out at least in triplicate.

Immunohistochemistry. Vessel development in cultured renal cortex explants was monitored by immunohistochemical methods. Cryosections (8 μm) were fixed and incubated with the endothelium specific antibody EC1 as described in detail previously (Kloth *et al.*, 1995).

RT-PCR analysis. Expression levels of GDNF were studied with a quantitative RT-PCR approach. A detailed description was published recently (Suter-Crazzolara and Unsicker, 1996). PCR products were separated by gel electrophoresis and analyzed by Southern hybridization. Signal intensities were analyzed with a Molecular Dynamics PhosphoImager. The signal intensity of the endogenous GDNF mRNA signal was determined and divided by that of the competitor internal standard RNA (IS-RNA).

RESULTS AND DISCUSSION

GDNF Stimulates Renal Blood Vessel Development

To analyze mechanisms by which GDNF may act during renal blood vessel development, the factor was applied to explants of the nephrogenic zone. To exclude effects of other growth factors present at varying concentrations in serum, a well-established serum-free culture model was employed. This model allows for long-term analysis of effects of growth factors on renal blood vessel development in an organ-specific context (Minuth *et al.*, 1992; Kloth *et al.*, 1995, 1998).

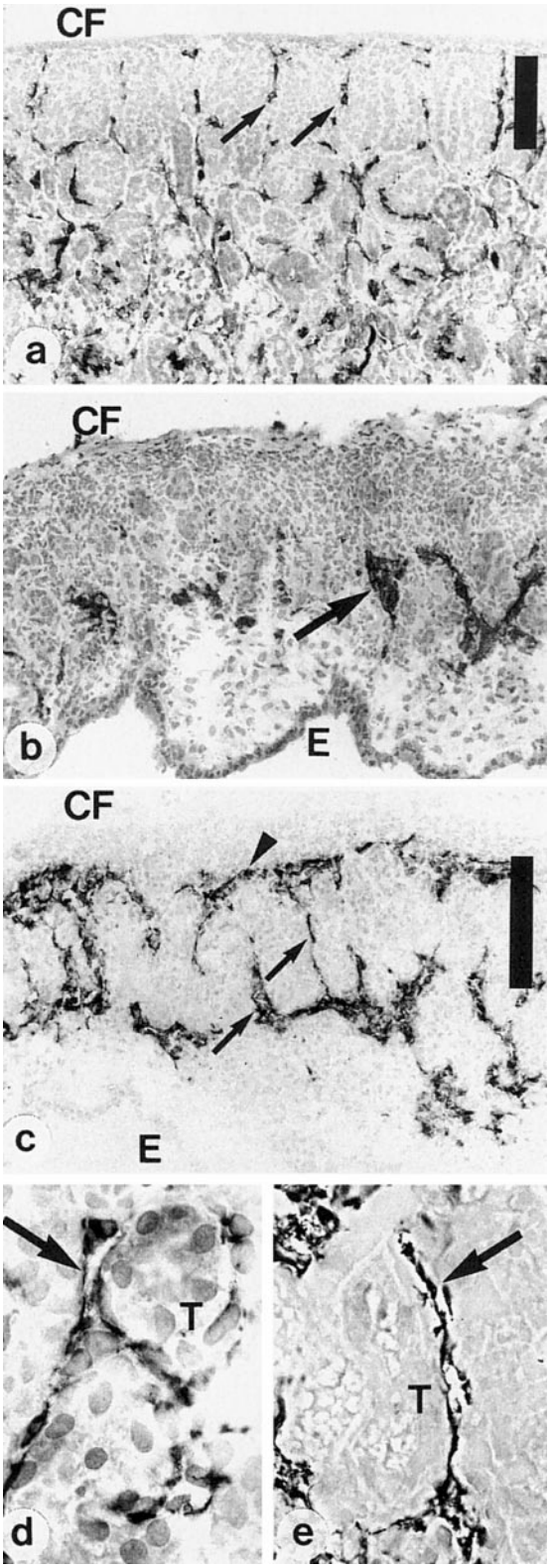
Thin blood vessels can be observed within the nephrogenic zone of the developing kidney (Kloth *et al.*, 1997). Characteristically, these vessels have lengths of 80 to 100 μm and run in a parallel fashion toward the organ capsule (Fig. 1a). In explants cultured for 13 days in serum-free basal medium, such blood vessels were not detected (Fig. 1b). These explants reveal clusters of EC1-immunoreactive endothelial cells, not found in the developing kidney or under culturing conditions in the presence of hormones (Kloth *et al.*, 1995). This indicates that serum-free medium lacks important regulatory signals, which are present *in situ*.

In contrast, explants cultured for 13 days in the presence of GDNF contain vessels that retained integrity and their typical spatial architecture. Following application of GDNF the vessels have broadened and elongated (Fig. 1c). Moreover, cultured explants have widened, and vessel-like structures of about 150 μm length are detected frequently. The mitogenic effect of GDNF was confirmed by comparing the amount of proliferating cells in GDNF-stimulated and control explants (data not shown). Addition of GDNF results in an enlarged vessel network which maintains its typical spatial organization even after 13 days of culture. GDNF therefore appears to mediate both mitogenic as well as morphogenic effects on the developing vessels. The factor stimulates vessel elongation within a regular framework, and the vessels retain their characteristic three-dimensional organization. These structures are strongly reminiscent of those found in the developing kidney *in situ* (Fig. 1a). Furthermore, small capillary-like, lumen-containing structures are a typical feature of GDNF-treated explants (Fig. 1e), but were never observed in explants cultured in basal medium alone.

GDNF mRNA Levels Are Not Significantly Altered by Stimulation of Cortex Explants with Hormones

In the past we have described several hormones that appear to have similar effects on vessel development as those described here for GDNF. These are aldosterone in combination with vitamin D_3 and retinoic acid (Kloth *et al.*, 1995, 1998). Therefore, we wanted to investigate whether GDNF may act as a mediator for these substances in this process. For this purpose, we have chosen a competitive RT-PCR approach that we have described in detail previously (Suter-Crazzolara and Unsicker, 1996).

GDNF mRNA can be detected in rabbit tissues with oligonucleotides specific for human and rat GDNF (Fig. 2, lanes 1 to 8). The L- and S-GDNF forms result



from an alternative splicing event within GDNF coding sequence (Suter-Crazzolara and Unsicker, 1994). GDNF mRNA can be detected both in explants of the nephrogenic zone (Fig. 2b, lane 1) as well as in the rabbit kidney cortex (data not shown).

Next, cortex explants were analyzed for GDNF mRNA expression levels after either aldosterone in combination with vitamin D_3 or retinoic acid treatment. None of the treatments resulted in an alteration of expression levels of GDNF mRNA. No significant differences in expression levels of GDNF mRNA can be observed between freshly prepared explants (Fig. 2b, lane 1), explants cultured in basal medium (lane 2), vehicle-treated explants (lanes 5 and 6), or medium supplemented with hormones (lanes 3, 4, 7, and 8). These results indicate that GDNF does not act as a mediator for either of these substances.

Our data do not allow an assessment whether the effects of GDNF are direct or indirect. However, no reports exist to date describing the presence of one of the known GDNF receptors on endothelial cells, which appears to suggest an indirect effect. The expression of the mRNAs for GDNF and its receptors by different cell population of the nephronic zone was demonstrated. While GDNF is produced by the mes-

FIG. 1. GDNF modulates renal blood vessel formation. Corticomedullary oriented sections of the kidney cortex and cultured renal explants are shown. Blood vessels in the nephrogenic zone of the neonatal rabbit kidney were visualized by employing the antibody EC1. CF, fibrous organ capsule; E, epithelium; T, tubules. (Magnification: a–c, 120×; d and e, 472×.) (a) Developing blood vessels of the cortical region (arrows) of neonatal rabbits can be observed that run in parallel towards the fibrous organ capsule. They are less than 10 μm in diameter and 80 to 100 μm in length. Black bar, 100 μm. (b) Explants cultured for 13 days in basal medium are characterized by the lack of blood vessels. Endothelial cells are arranged in large, undifferentiated clusters (arrow). (c) GDNF treated explants show vessel-like structures (arrows) of approximately 150 μm length. Vessel diameters range between 8 and 20 μm. Vessels are arranged in parallel, in a manner strikingly similar to that observed in a. They are connected by endothelial cells (arrowhead), a typical feature of the developing vascular system of the kidney. Black bar, 150 μm. (d) Enlargement of a typical capillary as found in a freshly prepared cortical explant. The vessel is surrounded by tubuli of the neonatal kidney. The small lumen (arrow) is lined by EC1-positive endothelial cells. (e) Capillaries (arrow) can be observed between tubular elements in GDNF-treated explant, after 13 days of culture.

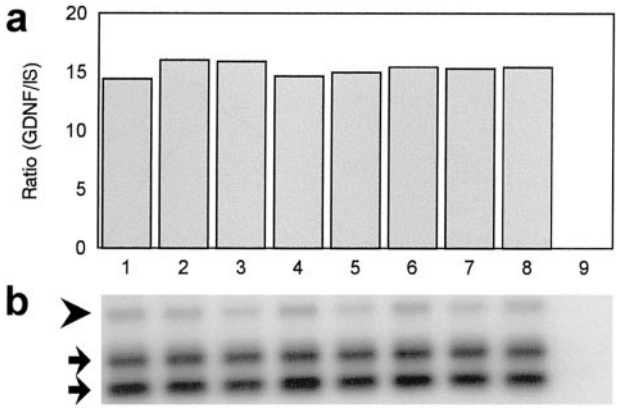


FIG. 2. Expression of the mRNA for GDNF in rabbit cortex explants, after treatment with various stimuli. Explants were prepared and cultured as described under Materials and Methods: Total RNA from explants was reverse transcribed in the presence of internal standard RNA (IS-RNA). C-DNA was amplified by RT-PCR with gene specific primers. (a) Analysis of the relative signal intensities of the IS-RNA and GDNF mRNA signals, observed in (b). (b) Indicated are the two forms of endogenous GDNF mRNA (arrows) and the position of the IS-RNA (arrow head). RNA was obtained from: lane 1 freshly prepared explants, lane 2 explants, cultured for 1 day in basic medium, lane 3 explants treated with aldosterone and vitamin D_3 for 1 day, or lane 4 two days. Lanes 5 and 6, explants pretreated (3 h) with basal medium and ethanol prior to 1 and 2 days (respectively) of culture in basal medium. Lanes 7 and 8, explants stimulated (3 h) with basal medium and retinoic acid, prior to 1 and 2 days of culture, respectively. Lane 9, negative control (RT-PCR in the absence of cDNA).

enchymal cells (Hellmich *et al.*, 1996; Suvanto *et al.*, 1996), the Ret receptor was detected on the epithelium of the uretric bud and the collecting duct ampullae (Pachnis *et al.*, 1993; Schuchardt *et al.*, 1996). GDNF appears to be an early acting factor essential for kidney development (reviewed by Sariola and Sainio, 1997).

It may well be that GDNF and the hormones used in this study employ independent pathways to modulate renal vascularization. On the other hand, the investigations of transgenic animals clearly document the superior importance of GDNF for renal development. Thus, we tend to speculate that GDNF is a higher order signal for modulating renal vascularization. The results of our tissue culture experiments carried out in an organotypic context provide first evidence that GDNF may be one of the factors responsible for the coordinated development of collecting ducts, nephrons, and blood vessels in the nephrogenic zone. It would be of main interest to investigate whether GDNF affects the expression of angiogenic factors such as VEGF or hormone receptors by the developing collecting ducts and nephrons.

ACKNOWLEDGMENTS

The skillful technical assistance of Elfriede Eckert, Christiane Wanke, and Ulrike Prüfer is gratefully acknowledged. Part of this work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG KL 741/7-1) and the Medical Faculty of the University of Heidelberg (Project 82/1995).

REFERENCES

Hellmich, H. L., Kos, L., Cho, E. S., Mahon, K. A., and Zimmer, A. (1996). Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech. Dev.* **54,** 95–105.

Kloth, S., Ebenbeck, C., Kubitza, M., Schmidbauer, A., Röckl, W., and Minuth, W. W. (1995). Stimulation of renal microvascular development under organotypic culture conditions. *FASEB J.* **9,** 963–967.

Kloth, S., Ebenbeck, C., Monzer, J., de Vries, U., and Minuth, W. W. (1997). Three dimensional organization of the developing vasculature of the kidney. *Cell Tissue Res.* **287,** 193–201.

Kloth, S., Gerdes, J., Wanke, C., and Minuth, W. W. (1998). Basic fibroblast growth factor (bFGF) is a morphogenic modulator in kidney vessel development. *Kidney Int.* **53,** 970–978.

Minuth, W. W., Dermietzel, R., Kloth, S., and Hennerkes, B. (1992). Construction of an apparatus for perfusion cell cultures which enables *in vitro* experiments under organotypic conditions. *Eur. J. Cell Biol.* **57,** 132–137.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382,** 76–79.

Pachnis, V., Mankoo, B., and Constantini, F. (1993). Expression of the cRet proto-oncogene during mouse embryogenesis. *Development* **119,** 1005–1017.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., and Westpaal, H. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382,** 73–76.

Roberts, A. B., and Sporn, M. B. (1990). The transforming growth factor-βs. *In* "Peptide Growth Factors and Their Receptors I" (M. B. Sporn, and A. B. Roberts, Eds.), pp. 419–472. Springer Verlag, Berlin / Heidelberg / New York.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He B., Lira, S. A., and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382,** 70–73.

Sariola, H., and Sainio, K. (1997). The tip-top branching ureter. *Curr. Opin. Cell Biol.* **9,** 877–884.

Schuchardt, A., D'Agati, V., Pachnis, V., and Constantini, F. (1996). Renal agenesis and hypodysplasia in ret-k-mutant mice result from defects in uretric bud development. *Development* **122,** 1919–1929.

Suter-Crazzolara, C., and Unsicker, K. (1994). GDNF is expressed in two forms in many tissues outside the CNS. *NeuroReport* **5,** 2486–24874.

Suter-Crazzolara, C., and Unsicker, K. (1995). Improved expression of toxic proteins in *E. coli*. *Biotechniques* **19,** 202–220.

Suter-Crazzolara, C., and Unsicker, K. (1996). FGF-2 induces GDNF mRNA levels in C6 glioma cells. *Mol. Brain Res.* **41,** 175–182.

Suvanto, P., Hiltunen, J. O., Arumäre, U., Moshnyakov, M., Sariola, H., Sainio, K., and Saarma, M. (1996). Localization of glia cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by *in situ* hybridization. *Eur. J. Neurosci.* **8,** 816–822.