Cross talk between primary human renal tubular cells and endothelial cells in cocultures

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Tasnim F, Zink D. Cross talk between primary human renal tubular cells and endothelial cells in cocultures. Am J Physiol Renal Physiol 302: F1055–F1062, 2012. First published February 8, 2012; doi:10.1152/ajprenal.00621.2011.—Interactions between renal tubular epithelial cells and adjacent endothelial cells are essential for normal renal functions but also play important roles in renal disease and repair. Here, we investigated cocultures of human primary renal proximal tubular cells (HPTC) and human primary endothelial cells to address the cross talk between these cell types. HPTC showed improved proliferation, marker gene expression, and enzyme activity in vitro. Also, the long-term maintenance of epithelia formed by HPTC was improved, which was due to the secretion of transforming growth factor-β1 and its antagonist α2-macroglobulin. HPTC induced endothelial cells to secrete increased amounts of these factors, which balanced each other functionally and only displayed in combination the observed positive effects. In addition, in the presence of HPTC endothelial cells expressed increased amounts of hepatocyte growth factor and vascular endothelial growth factor, which have well-characterized effects on renal tubular epithelial cells as well as on endothelial cells. Together, the results showed that HPTC stimulated endothelial cells to express a functionally balanced combination of various factors, which in turn improved the performance of HPTC. The results give new insights into the cross talk between renal epithelial and endothelial cells and suggest that cocultures could be also useful models for the analysis of cellular communication in renal disease and repair. Furthermore, the characterization of defined microenvironments, which positively affect HPTC, will be helpful for improving the performance of this cell type in vitro applications including in vitro toxicology and kidney tissue engineering.

primary human renal proximal tubular cells; transforming growth factor-β; α2-macroglobulin; defined microenvironment; communication of renal cells

THE EPITHELIAL CELLS OF THE renal tubule perform important metabolic and endocrinological functions and play essential roles in renal drug, water, and solute transport (2, 39). Due to their functions in glomerular filtrate concentration and drug transport in particular, renal proximal tubular cells are a major target for drug-induced toxicity. Therefore, this cell type is important for in vitro toxicology (1, 20, 22, 40), but approved in vitro models based on renal cells have not yet been developed. One major obstacle is cellular dedifferentiation under in vitro conditions, which is observed in primary cells as well as in cell lines (1, 20, 40).

In the kidney, the renal tubules are morphologically and functionally linked with the peritubular capillaries, which is essential for renal transport and reabsorption functions and oxygen supply (2, 17). Although the endothelial cells of the peritubular capillaries and the renal tubular epithelial cells are closely apposed, they are separated by the tubular basement membrane, a narrow interstitial region containing microfibrils, and the capillary basement membrane. Thus it would be expected that these two cell types communicate mainly via soluble factors.

The tubular epithelium and the peritubular endothelium are not only functionally linked under normal conditions, but both also play key roles in diabetic and nondiabetic kidney disease (11, 17, 21, 29, 37). To better understand such normal and pathological interactions, it would be helpful to further characterize the communication between the respective epithelial and endothelial cell types and the factors involved. Recent data suggest that vascular endothelial growth factor (VEGF) signaling is important in the communication between renal tubular epithelial and peritubular endothelial cells and is involved in the development of age-related nephropathy and the progression of chronic kidney disease (9, 12, 25).

To further study the communication between renal tubular epithelial and endothelial cells and the factors involved, coculture systems are useful. Studying coculture systems might also help to identify factors that improve renal cell performance in vitro. Similar to renal epithelial cells, primary hepatocytes readily dedifferentiate under in vitro conditions, which is also one of the major obstacles here for applications in in vitro toxicology. Hepatocyte functions can be improved in cocultures with endothelial cells (24, 28). One major disadvantage of coculture systems is that they are poorly defined and standardization is difficult. Thus, to create defined microenvironments, it would be helpful to identify the soluble factors secreted by endothelial cells that improve the performance of other cell types.

Identification of such factors might also help to improve the performance of renal and other organ-specific cell types in other in vitro applications. For instance, renal proximal tubular cells are also important for kidney tissue engineering, and the development of bioartificial kidneys is one major application (23, 30, 34). Also, in this application it would be of primary importance to inhibit dedifferentiation of renal tubular cells. In addition, it would be critical to maintain differentiated epithelia during prolonged time periods.

Here, we investigated the cross talk between human primary endothelial cells and human primary renal proximal tubular cells (HPTC) in cocultures. The results showed that HPTC stimulated the endothelial cells to generate a special microenvironment of secreted soluble factors which, in turn, improved HPTC performance.

MATERIALS AND METHODS

Cell culture. HPTC were obtained from ScienCell Research Laboratories (Carlsbad, CA) and the American Type Culture Collection (ATCC, Manassas, VA) and were cultivated as described (18). Pri-
Innovative Methodology

Table 1. Details of primer pairs used for the qPCR analysis of HUVEC gene expression

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer Pairs</th>
<th>Amplicon, bp</th>
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<tr>
<td>VEGF-A</td>
<td>F'5'-GCAGCAATGGCAGAAGGGG-3'</td>
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<tr>
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<td>R'5'-TTCGAAATGCGTTTGAGG-3'</td>
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<tr>
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<td>R'5'-GGGAGATAGTATAGACTATCCATG-3'</td>
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<td>VEGF-B</td>
<td>F'5'-AGAACCGAAAAACCTTAAC-3'</td>
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<td>R'5'-TAAAGATGACGGAAGAAGA-3'</td>
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<td>R'5'-TCTTTTACGCTAACATACAA-3'</td>
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<td>VEGF-C</td>
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Sequences of primer pairs (forward (F) and reverse (R)) are shown. qPCR, quantitative real-time PCR; HUVEC, human umbilical vein endothelial cells. See the text for definitions of markers. The sizes of the amplicons are provided in base pairs (bp).

Determination of cell numbers and immunostaining. Cell counting and immunostaining were performed as described (44).

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) was performed as described (31). Primer sequences for genes analyzed in endothelial cells are provided in Table 1. Primer sequences for renal marker genes are described elsewhere (31). The expression levels of all marker genes were normalized to the expression levels of GAPDH to account for differences in cell numbers.

Determination of γ-glutamyl transferase activity. γ-Glutamyl transferase (GTT) activity was determined as described (31).

ELISA. The levels of transforming growth factor (TGF)-β1 and α2-macroglobulin (A2M) in the culture medium were quantified by using ELISA kits specific for human TGF-β1 (Abfrontier, Seoul, Korea) or A2M (Abnova, Taipei, Taiwan). The assays were performed according to the manufacturers’ instructions.

Table 2. Numbers of independent experiments and numbers of replicates for each independent experiment

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<th>Figure Related to Experiments</th>
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Column 2 (no. of independent experiments) indicates how many independent experiments were performed for each particular type of experiment (note that some figures summarize different types of experiments). When different types of experiments were performed in relation to a particular figure, the numbers of independent experiments for each type of experiment were similar. In cases where independent experiments were performed twice, no significant differences were observed between the results of the independent experiments.
immonstaining of the tight junctional protein zonula occludens (ZO)-1 (Fig. 1, E and F). Tight junctions were more extensive and regular in the lower compartment (Fig. 1, E and F). Cells in this compartment resided on stiff tissue culture polystyrene (TCPS), whereas they were kept on more compliant polyester membranes in the upper compartment. These findings were consistent with our results showing that HPTC performed better on TCPS and other stiff materials compared with more compliant membrane materials (19). Therefore, in all of the following experiments HPTC were kept in the lower compartment.

Next, we assessed the influence of HUVEC on HPTC gene expression patterns. HPTC were cultivated in mono- or cocultures and after 4 days the expression of 18 HPTC marker genes was assessed by qPCR. The markers tested included the genes for the adherens junction proteins E-cadherin (E-CAD) and N-cadherin (N-CAD) and ZO-1, as well as the genes for the brush-border enzymes GGT and CD13 (aminopeptidase N). Furthermore, the genes for the water channel aquaporin-1 (AQP1), the 25-hydroxyvitamin D3 1α-hydroxylase (Vit D3 Hydr), and the multidrug resistance gene (MDR1) were included. Also, the expression of genes encoding the following transporters was assessed: organic anion transporter 1 (OAT1), OAT3, organic cation transporter 1 (OCT1), organic cation/ carnitine transporter 2 (OCTN2), glucose transporter 5 (GLUT5), Na\(^+\)-K\(^+\)-ATPase, Na\(^+\)-HCO\(_3\)\(^-\) cotransporter 1 (NBC1), sodium-dependent glucose cotransporter 2 (SGLT2), and proton-coupled peptide transporter 1 (PEPT1) and PEPT2. We also determined expression of the myofibroblast marker α-smooth muscle actin (α-SMA) and the mesenchymal cell marker vimentin.

Figure 2 shows that the expression levels of α-SMA and vimetin as well as of the epithelial junctional proteins remained unchanged in cocultures (compared with monocultures). Of the 15 remaining HPTC-specific markers, 10 were significantly upregulated, whereas the expression levels of the other 5 markers were not significantly changed. Overall, this result showed that epithelial and mesenchymal markers remained unchanged, whereas the majority of HPTC-specific markers was upregulated. The highest levels of upregulation were observed in case of genes encoding for transporters and channels. Thus OAT1 was upregulated ~5-fold, and OAT3, GLUT5, and AQP1 were upregulated ~2.5-fold. NBC1 and SGLT2 were upregulated about twofold. The levels of upregulation of OCT1 and brush border enzymes (CD13 and GGT) were less than twofold.

To confirm the observed upregulation of gene expression at a functional level, we determined the activity of the brush-border enzyme GGT in mono- and cocultures of HPTC (Fig. 3). HPTC were cocultivated either with HUVEC or with HRGEC to exclude that the observed effects of endothelial cells were specific for HUVEC. In both cocultures GGT activity was ~1.5-fold higher than in monocultures (Fig. 3). This is in agreement with the observed upregulation of the GGT gene as determined by qPCR (Fig. 2).

Furthermore, we investigated cell proliferation in mono- and cocultures. The presence of HPTC had no significant influence on the numbers of HUVEC or HRGEC (Fig. 4A). In contrast, significantly higher numbers of HPTC were obtained in cocultures with HUVEC or HRGEC compared with monocultures [note that the GGT activity (Fig. 3) was normalized to cell numbers, and gene expression levels (Fig. 2) were normalized to GAPDH to account for differences in cell numbers].

In summary, endothelial cells influenced different variables of HPTC performance. These included cell numbers, gene expression patterns, and enzyme activity. In addition, endothelial cells inhibited the TGF-β1-dependent formation of cell aggregates and the disruption of renal epithelia in long-term cultures. Overall, the performance of HPTC was improved in cocultures.

Cross talk between HPTC and HUVEC and soluble factors secreted by HUVEC. As HPTC and endothelial cells were not in direct contact in our coculture system, it was likely that the effects of endothelial cells on HPTC were mediated by secreted soluble factors. First, we performed qPCR analysis to identify such factors expressed by HUVEC. This analysis also addressed the influence of HPTC on their expression.

The qPCR analysis was performed with HUVEC, which had been kept in monocultures or which had been cocultivated with HPTC for 4 days (Fig. 5). The expression of the genes for the following secreted factors was analyzed: VEGF-A, -B, and -C, fibroblast growth factor (FGF)-2, insulin-like growth factor (IGF)-1, hepatocyte growth factor (HGF), TGF-β1, and platelet-derived growth factor (PDGF-A and PDGF-B). All of these factors are known to be secreted by endothelial cells and/or have effects on renal tubular cells. In addition, we analyzed the expression levels of the genes for the following receptors: VEGFR-2, FGFR-2, and TGF-βR1. Figure 5 shows that the
levels of VEGFR-2 and TGF-β1 were similar in mono- and cocultures, whereas the level of FGFR-2 expression was upregulated (~3-fold). IGF-1 and PDGF-B expression was similar in mono- and cocultures. PDGF-A was downregulated. FGF-2 mRNA expression was upregulated, but increased secreted FGF-2 protein levels could not be detected by ELISA (data not shown). However, there was a marked (>2-fold) upregulation of VEGF-A, VEGF-B, and HGF. These factors are potent regulators of endothelial and renal tubular epithelial cells (5, 8, 13–16, 26, 32, 33, 35, 41).

Surprisingly, TGF-β1 showed the largest increase in expression levels, which were ~4.5-fold higher in cocultures. This result did not appear to be in agreement with the previous findings, which showed inhibition of TGF-β1-dependent effects in cocultures, such as cell aggregate formation and disintegration of epithelia (Fig. 1). Also, improved cell type-specific differentiation (as revealed by marker gene expression, Fig. 2) and increased HPTC proliferation (Fig. 4) in cocultures did not seem to be in agreement with the observed upregulation of TGF-β1, as TGF-β1 has known antiproliferative effects (7) and induces transdifferentiation of renal epithelial cells (4, 42).

Therefore, we also addressed the expression levels of the natural TGF-β1 antagonists A2M, decorin (DEC), follistatin (FST), and follistatin-like 3 (FSTL3). FSTL3 was significantly downregulated (Fig. 5), whereas FST and DEC were significantly but only slightly upregulated (<2-fold). In contrast, A2M was strongly upregulated. Intriguingly, both A2M and TGF-β1 were upregulated in similar ways and in both cases the expression levels were ~4.5-fold higher in cocultures (Fig. 5). This suggested that A2M was the major antagonist of TGF-β1

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**Fig. 2.** Gene expression levels of HPTC determined by quantitative real-time PCR (qPCR). The relative expression levels of the genes indicated (x-axis) were assessed with HPTC kept for 4 days in monocolures (light grey bars) or cocultures (dark grey bars). Values are means ± SD, and the mean values obtained with monocolures were set to 1. *Significant differences between mono- and cocultures (P < 0.05).

**Fig. 3.** γ-Glutamyl transferase (GGT) activity of HPTC. HPTC were kept for 3 days in monocolures or in cocultures with either human umbilical vein endothelial cells (HUVEC) or human renal glomerular endothelial cells (HRGEC). The medium was supplemented with GGT substrates, and after 1 h the concentration of the reaction product p-nitroaniline (nmol/ml) was determined. The results were normalized to the cell numbers in each sample, which were counted after the assay. Values are means ± SD. *Significant differences between mono- and cocultures (P < 0.05).

**Fig. 4.** Cell numbers. The numbers of endothelial cells (A) or HPTC (B) were determined by cell counting on day 3 after cell seeding. The bars indicate the numbers of the respective cell types per Transwell compartment (means ± SD). A: HUVEC or HRGEC were cultivated in monocolures (light grey bars, the same type of endothelial cell in both compartments) or cocultures with HPTC (dark grey bars). B: HPTC were cultivated in monocolures or cocultures with HUVEC or HRGEC. *Significant differences between mono- and cocultures (P < 0.05).
in the coculture system used here and that the inhibition of TGF-β1-dependent effects observed in the cocultures was due to the expression of A2M.

TGF-β1 and its antagonist A2M regulate maintenance of renal epithelia. To address this hypothesis, we first determined the levels of secreted TGF-β1 and A2M in the supernatants of mono- and cocultures by ELISA. TGF-β1 levels were determined separately in the HPTC and HUVEC compartments of cocultures. The results (Fig. 6A) confirmed that TGF-β1 levels were elevated in cocultures and that HUVEC were the major source of TGF-β1. Furthermore, the results confirmed that TGF-β1 secretion by HUVEC was significantly increased in the presence of HPTC. In addition, the results also confirmed that A2M secretion by HUVEC was significantly increased in the presence of HPTC (Fig. 6B).

To address the effects of such increased levels of TGF-β1 and A2M at a functional level, we added similar concentrations of these factors (as found in cocultures) to monocultures of HPTC. For these experiments, HPTC monocultures were kept in normal multiwell plates and either hr TGF-β1 or hr A2M or a combination of both factors was added. Control cells were left untreated. Figure 7 shows the results obtained 2 wk after cell seeding. Variable results were obtained with control cells (Fig. 7A), and some of the wells showed the typical cell aggregates induced by TGF-β1 (note that also HPTC secrete some TGF-β1; see Fig. 6A and Ref. 43). In these wells, cell-depleted areas were also observed, which were due to reorganization of the epithelium after formation of a closed epithelial sheet. In hr TGF-β1-treated cells, these processes were enhanced and all of the wells treated with hr TGF-β1 consistently showed cell aggregates and major rearrangements of the epithelium (Fig. 7B). Also, in all of the wells treated with hr A2M, large-scale rearrangements of the epithelium were observed, leading to cell-depleted areas. However, cell aggregates were absent (Fig. 7D). When HPTC were treated with a combination of hr TGF-β1 and hr A2M (Fig. 7C), cell aggregates as well as large-scale rearrangements were absent and the epithelium was well preserved.

In summary, the results showed that prolonged maintenance of the epithelium formed by HPTC and inhibition of cell aggregate formation and epithelial rearrangements, as observed in cocultures with HUVEC (Fig. 1), were due to the combined effects of TGF-β1 and A2M.

DISCUSSION

Our results showed that endothelial cells have a profound impact on the performance of HPTC in cocultures by generat-
numbers of endothelial cells in the presence of HPTC (Fig. A2M (38). Nevertheless, the results did not show any increased
enerative effects on endothelial cells, which can be neutralized by
pathway 11 has been shown to have antiprolif-
endothelial cells. TGF-
pathway 11 and its antagonist A2M.

Altogether, the presence of the different factors identified here
ing a special microenvironment. This microenvironment had
positive effects on HPTC numbers, cell type-specific gene
expression, enzyme activity, and the maintenance of differenti-
tiated epithelia during extended time periods. Characteristic of
the microenvironment generated by the endothelial cells were
increased amounts of VEGF, HGF, TGF-β1, and its antagonist
A2M. HPTC stimulated the production of these factors by
endothelial cells, which in turn improved the performance of
HPTC (Fig. 8).

HGF and VEGF stimulate growth and survival of renal
tubular epithelial cells (8, 13, 35). In contrast, TGF-β1 pro-
motes epithelial-to-mesenchymal transition (EMT) of this cell
type (4, 42) and has antiproliferative effects (7). However,
VEGF inhibits TGF-β1-induced EMT of renal tubular epithe-
lium cells (10). TFG-β1-induced effects were also inhibited by
A2M in the microenvironment created by endothelial cells.

Alternatively, the presence of the different factors identified here
can explain the positive effects of cocultures on HPTC cell
numbers, differentiation, and improved long-term maintenance
of differentiated epithilia. The experimental results directly
demonstrated that improved maintenance of epithelia was due
to the combined effects of TGF-β1 and its antagonist A2M.

The factors identified here could potentially also affect
endothelial cells. TGF-β1 has been shown to have antiprolif-
erative effects on endothelial cells, which can be neutralized by
A2M (38). Nevertheless, the results did not show any increased
numbers of endothelial cells in the presence of HPTC (Fig.

Fig. 8. Summary of the interactions between HPTC and endothelial cells in
cocultures. The presence of HPTC stimulated endothelial cells (grey arrow,
right), which expressed increased amounts of hepatocyte growth factor (HGF)
and vascular endothelial growth factor (VEGF) in cocultures. HGF and VEGF
have well-characterized effects on both cell types (inner circle of arrows).

Endothelial cells also secreted increased amounts of TGF-β1 and its antagonist
A2M in the presence HPTC (grey arrows, left). A2M balanced the effects of
TGF-β1, as illustrated by the Yin-Yang symbol, and the long-term mainte-
nance of renal epithelia was improved in the presence of both factors.

Cocultivation with endothelial cells also improved HPTC proliferation and
differentiation.
experiments, and the HPTC response to HRGEC and HUVEC was always similar. Thus the effects did not appear to be specific for particular types of endothelial cells.

Irrespective of the in vivo situation, the results obtained here will be valuable for improving HPTC performance under in vitro conditions. In this respect, it is important to note that the expression levels of OAT1, OAT3, and OCT1 and of various other transporters were substantially increased in cocultures. Dedifferentiation and low expression levels of drug transporters, which renders the cells insensitive to drug exposure, is a particular problem in in vitro nephrotoxicology (1, 20). The analysis of the coculture microenvironment performed here will help to establish improved and more defined in vitro culture systems.

In bioartificial kidneys, it is important to maintain a differentiated HPTC epithelium during prolonged time periods (23, 30), and also in this regard the results obtained here will be helpful. It is interesting to note that we obtained previously similar effects, as observed here in the presence of A2M, with another TGF-β1 antagonist, namely, bone morphogenetic protein (BMP)-7 (31). This confirms the central importance of antagonizing TGF-β1-induced effects, at least in vitro.

One interesting question is why endothelial cells upregulated both TGF-β1 as well as A2M. TGF-β1 plays in vivo important roles in coordinating the response to injury and inflammation, and TGF-β1 expression is consistently increased in different cell types in such situations. However, overexpressing TGF-β1 activity has deleterious effects. Probably, in the in vivo situation a delicate balance is achieved between TGF-β secreted by various sources and their antagonists, which could be also generated by various sources. One candidate for additional factors that could be involved in vivo is the TGF-β1 antagonist BMP-7. Although BMP-7 did not play a role in the model system investigated here, it is secreted in the adult kidney by several cell types, including collecting duct cells and podocytes (6, 27, 36).

It is also worth mentioning that A2M binds not only to TGF-β1 but to a large variety of growth factors and proteases, which could be important in vivo. Also, binding to other factors in the HPTC medium in the absence of increased levels of TGF-β1 might explain why the HPTC epithelium was disrupted when the cells were treated with hr A2M only.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: F.T. and D.Z. provided conception and design of research; F.T. performed experiments; F.T. analyzed data; F.T. and D.Z. interpreted results of experiments; F.T. prepared figures; D.Z. drafted manuscript; D.Z. edited and revised manuscript; D.Z. approved final version of manuscript.

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Selective stimulation of VEGFR2 accelerates progressive renal disease. 


