SUMMARY AND DISCUSSION

The purpose of this thesis was to investigate the interactions of humoral and cellular factors with glomerular epithelial cells in culture to gain insight in the pathogenesis of GVEC dedifferentiation and detachment *in vivo*. Our study is composed of three parts. At first, we defined GVEC (de)differentiation by studying the structure and composition of the cells both *in vivo* and *in vitro* (chapter 1) as well as the adhesive characteristics of cultured GVEC (chapter 2). In close alignment, we tried to manipulate this (de)differentiation process by studying the effect of a physical parameter (hydrostatic pressure) (chapter 3). Secondly, we investigated in what way toxic (chapter 4) and inflammatory (chapter 5) factors can cause GVEC dedifferentation. Finally, we investigated the involvement of podocytes in necrotizing crescentic glomerulonephritis, to ascertain their potentially active role in glomerular inflammation (chapter 6).

Glomerular visceral epithelial cell structure and function

There has been considerable discussion over the years on the exact role of podocytes in maintaining the size and charge selective barrier function of the glomerular capillary wall. Although there is a strong association between podocyte dedifferentiation and proteinuria [48], this does not provide insight in causal relationships. At the moment, there is general agreement on the role of the negatively charged matrix network of the GBM as the main size and charge selective barrier to retain macromolecules in the circulation [107]. Neutralization of the negative charge of the GBM by anti-heparan sulphate side chain antibodies (JM-403)[221] leads to selective proteinuria without disturbance of podocyte morphology, emphasizing the importance of the negatively charged GBM. The adjustable collective width of the slit-pores to control hydraulic conductivity and water flow seems to be the major function of the podocytes [8,31,107,116,129], although the negatively charged cell surface probably also takes part in the charge selective barrier function of the glomerular capillary wall [119].

Attempts to further explore GVEC function often make use of cell culture techniques, but there is controversy on the identity of the cultured cells [94,166,236]. It was therefore of considerable importance to firmly establish the visceral epithelial phenotype of the cells we studied. By using the same techniques and antibodies which have been claimed as the most reliable tools to identify or disqualify cultured renal cells as real podocytes [94,188], we demonstrated that our cells were indeed glomerular visceral epithelial cells (chapter 1). As such, chapter 1 may serve as a guideline to characterize glomerular visceral epithelial cell cultures by their phenotype. In addition, a vast number of other properties have been described in the literature that contribute to our understanding of podocyte function under normal and pathological conditions (Table 1). However, extrapolation of glomerular epithelial cell function *in vitro* to the *in vivo* situation is hampered by their dedifferentiated morphology in culture.

Table 1: Various pathophysiological properties of podocytes of different species as decribed in the literature

Podocyte function	Examples of *in vivo* and *in vitro* findings [Literature]
ECM turnover	
Production	collagen [219]; laminin, fibronectin, proteoglycans [121,162]
Degradation	metalloproteinase [104]; gelatinase [232]
Fibrinolysis	u-PA/t-PA/PAI-1 production [91,100,190]; thrombin response [88,89,91]
Complement activation	
C-receptor expression	CR1 [10,183]; CR2 [110]; αvß3 (S-protein receptor) [46]
Production C-factors	C3 [194]; C4 [249]
Production inhibitors	DAF [184]; GCRF [181]; CD59 (protectin)[191]
Vasoactivity	
Response to factors	ANP [12,202]; ADH [137]; angiotensin II [103,202]; histamin/ bradykinin [175]; endothelin-1 [90], eicosanoids [52]; nucleotides [13,176,206]
Production of factors	nucleotidase activity [179,209]; endothelin-1/3 [54,109]; VPF [37], leukotrienes [14,86,180]; aminopeptidase A/N [15,209]
Proliferation	
Growth factors receptors	bFGF [214]; EGF [5,50,52]; TGF-ß [139,162]; IGF [5]; HGF [85]
Paracrine stimulation	PDGF [68]; heparin [39]
Lipid uptake	LDL/IDL [79,126,186]
Chemotaxis	IP-10 [74]; MCP-1 [198]; PMN-chemotactic factor [233]; complement (see above)
Immunological respons	
Immunoglobulin receptors	Fc receptor [7]
Cytokine receptors	IL-1 [87], IL-4 (?, chapter 5), IFN-γ/IFN-α (chapter 5 & 6)[146]

While there is clearly a structural and functional resemblance between podocytes of different species [46], one should compare experiments with different species with caution. Also, some of the epithelial cell preparations may not have been optimally characterized (see chapter 1).

Chapter 3 identifies hydrostatic pressure as a physical parameter which may be an important additional parameter of podocyte morphology and differentiation *in vivo* [9,27]. If the regulation of water flow, to which the podocytes are exposed *in vivo*, is an important function of the cells, then this flow may influence cultured podocytes as well. Indeed, GVEC cultured under basolateral hydrostatic pressure demonstrated whirl-like patterns of thrown-up, rounded cells and more importantly, a significant decrease of the aberrantly expressed cytokeratin-18 filaments was found. When exposed to physiological flow conditions some GVEC showed widening of cell-cell contacts and basolateral tight junctions which resembled morphologically early stages of podocyte (de)differentiation as seen in embryonic development [151,187] and in some renal diseases [48,129,187]. Pressure-induced morphological adaptation of cultured GVEC was associated with changes in functional characteristics of the podocytes as indicated by their difference in sensitivity to the toxic and immunological effects of IFN-γ. The hydrostatic pressure experiments demonstrated that cultured GVEC can be triggered to differentiate up to certain stages and their considerably altered physiology suggests that this culture system can be quite relevant for future studies with cultured GVEC. Using our hydrostatic pressure culture system, we were not able to achieve 'terminal' differentiation, i.e absence of proliferation [159,174] and strong expression of podocyte specific molecules such as podocalyxin [119], pp44 [158] and O-acetylated GD3 [188]. This implies that other factors as described below are also important for ultimate differentiation.

Factors affecting podocyte differentiation

At least three strongly interrelated factors determine the differentiated phenotype of normal podocytes *in vivo*: the unique polarized morphology with interdigitating foot processes between adjacent cells, the 250 Å wide specialized cell-cell contacts also called slit-pore membranes, and the strongly negatively charged (apical) cell membrane. Additionally, ultrafiltration of solute probably plays a role in terminal differentiation as well (chapter 3). In human and rat glomerular diseases and under experimental conditions *in vivo* and *in vitro*, affection of either one of these three factors inevitably leads to alterations in the other factors resulting in dedifferentiation and proteinuria, and sometimes in proliferation [68,159]. The functional and structural components of the podocyte as depicted in figure 1 probably act in concert to maintain the morphological determinants mentioned above. The mechanisms of podocyte dedifferentiation are schematically represented in figure 1 and discussed below by using examples of several experimental models.

At first, maintenance of the polarized podocyte morphology under the high flows and pressures that exist *in vivo* obviously requires strong cell-matrix (chapter 2) as well as cell-cell adhesion. That circulating (auto)antibodies directed to molecules on the interface between GVEC and the GBM, such as laminin [17,18,143], gp330 [117] or β_1-integrins [4,169], can lead to detachment of the GVEC and proteinuria, is a tempting but too simple pathway to explain proteinuria (Figure 1a). Most of the antibodies that cause proteinuria upon injection or perfusion *in vivo* are complement fixing [22,182,207] or (indirectly) activate the immune system in various ways [82,112,226], leading to additional damage to the GVEC or the GBM. Experiments with nephritogenic antibodies in naive recipients as performed by Aten et al. [17], Miettinen et al. [57] and Mendrick and Rennke [147] have confirmed that additional (immunological) factors

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are neccesary to cause proteinuria. In this light, foot process obliteration leading to total coverage of the glomerular capillary wall by GVEC cellular processes as seen in most renal diseases is hard to bring into agreement with the existence of massive proteinuria [31]. The morphological appearance of foot process obliteration suggests that glomerular epithelial cell-matrix adhesion is not, or only locally affected. Recent experiments of Kurihara et.al. have demonstrated that in protamine suphate or puromycin aminonucleoside treated nephrotic rats, the tight junctions are discontinuous and do not constitute a continuous barrier (Figure 1c), thus allowing the leakage of fluid at these sites [129]. These experiments point towards an association of GVEC cell-cell and not cell-matrix adhesion with the development of proteinuria.

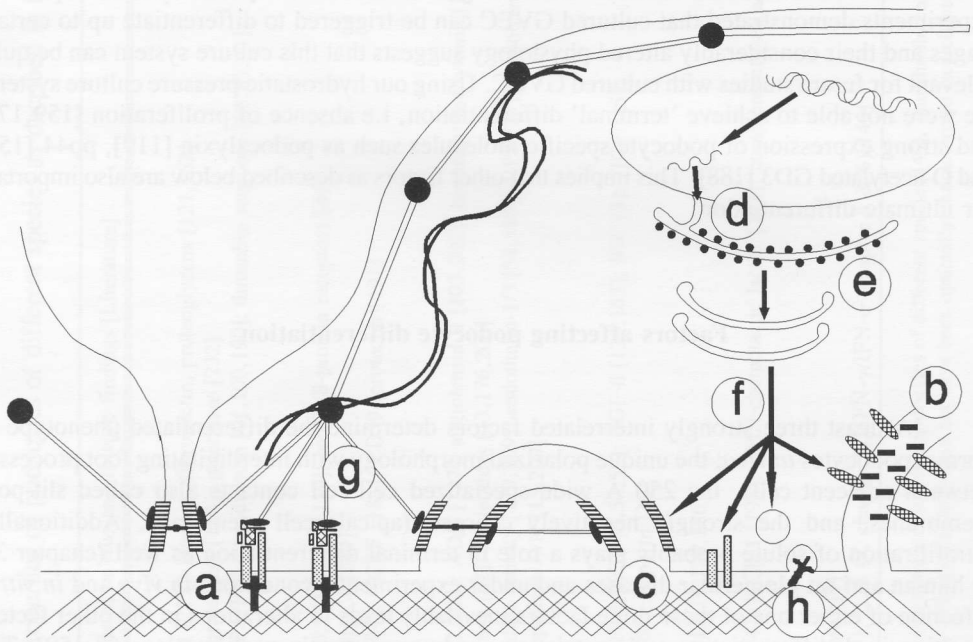

Figure 1: Factors involved in the differentiation of podocytes. Structural components are in the left half of the figure, with complex cytoskeletal, extracellular matrix and integrin interactions. In the right half, (macro) molecule synthesis and trafficking are visualized. The letters in circles refer to the mechanisms of dedifferentiation that are discussed in the text.

As emphasized earlier, cell-cell adhesion as visualized by tight junctions in immature and by slit pores in mature podocytes is crucial for compartmentalization and cellular physiology [152]. We showed that cell-cell adhesion of cultured GVEC as visualized by ZO-1 immunofluorescence [128] can be disturbed by the cytokine IFN-γ, which directly affected the transepithelial resistance without disturbing monolayer permeability for the 44 kD protein horse radish peroxidase (chapter 5; figure 1c). Since the molecules essential for maintenance of cell-cell adhesion such as ZO-1 appear to be present in large intracellular pools [129], this

process can be expected to be rapidly reversible upon withdrawal of the toxic agent, which was confirmed by our experiments (chapter 5). Disturbed cell-cell contacts and a lack of polarization can lead to disturbed intra- and extracellular protein transport and trafficking and to lateral diffusion of transmembrane proteins that are otherwise exlusively present in one of the compartments (Figure 1f)[32,156,163]. Whether these phenomena have contributed to the apical expression of otherwise basolaterally present MHC class I in IFN-γ treated GVEC cultures is unknown, but the similarly increased membrane domain-restricted expression of MHC class II and ICAM-1 do not support this observation (chapter 6). It is conceivable that this disturbed cellular physiology is essential in rendering the cells more vulnerable to (auto)antibodies interfering with cell-matrix adhesion. This process may also contribute to antibody-mediated and antigen-driven autoimmune phenomena by exposure of otherwise 'hidden' antigens to the basal, blood-exposed side of the GVEC.

The highly negative charge of the podocyte apical cell-membrane *in vivo* is thought to be neccesary as a repellant force between the cells [116,129,187] (Figure 1b). This negative charge in combination with fluid permeation through the GBM (chapter 3) will thus lead to a predominant intercellular route of fluid transport. Neutralization of this repelling negative charge by protamine sulphate [116,129] in rats leads to immediate (< 15 minutes) foot process obliteration and formation of tight junctions without a significant increase in proteinuria [149]. These experiments further support the role of the GBM in the size and charge selective barrier function of the capillary wall. This neutralization does not functionally disturb the membrane polarization which is maintained by degenerated slit pores (tight junctions)[129], as evidenced by podocalyxin which was confined to the apical cell membrane. In the experiments of Messina et.al. [149] some animals became oliguric after perfusion of protamine sulphate, confirming the importance of the epithelial cells with open slit pores in the regulation of water filtration.

There are several examples of disease models that are suspected to interfere with a number of cellular processes such as protein synthesis (Figure 1d), posttranslational processing (Figure 1e), molecular transport (Figure 1f), polymerisation and complexation of structural and functional molecules (Figure 1g) and endo- or exocytosis (Figure 1h). Oxygen radical species as detected in a variety of renal diseases [36,164,178] can lead to enhanced proteolytic degradation, production of eicosanoids, altered matrix composition and altered cell-membrane lipid composition [107,164]. Reactive oxygen metabolites can thus cause significant morphological, functional and biochemical alterations of the cellular and matrix determinants of glomerular ultrafiltration. Nephrotoxic agents such as puromycin aminonucleoside [223] and adriamycin [25] also cause a variety of GVEC changes *in vivo* and *in vitro*. Both agents interfered dose and time dependently with GVEC adhesion and proliferation (chapter 4). Although GVEC treated with these nephrotoxins demonstrated significantly decreased cytoskeletal, extracellular matrix and integrin immunoreactivity, this could not be explained by decreased protein synthesis (chapter 4). The mechanism of action of these agents is probably through the (indirect) formation of oxygen radicals [107], but effects on posttranslational processing of proteins [78,120] or interference with cellmembrane fluidity [66,172] might also play a role in their pathogenesis (chapter 4).

The role of proteinuria itself on degeneration and dedifferentiation of GVEC, is still a matter of discussion. The pathway through which this proteinuria-induced GVEC damage may lead to glomerulosclerosis is also hypothetical [242]. Experimental models of both Adriamycin [171] and puromycin nephrosis [1] in Nagase analbuminemic rats have demonstrated identical GVEC changes without induction of proteinuria, as compared to SD rats with heavy proteinuria. These experiments indicated that the GVEC was initially damaged after which marked proteinuria

occurs, which was confirmed by Messina et al. [148]. The exposure to high amounts of protein probably leads to additional toxic effects contributing to the altered and dedifferentiated state of the cells [153]. Healthy rats made proteinuric by repeated injections with homologous serum albumin showed mild GVEC changes without effacement or decreased endocytic function, implicating that proteinuria itself is not a causal factor in the development of glomerular sclerosis [200]. In models using high amounts of foreign protein injected into the animals, an effect on the GVEC via the immune system can not be excluded [30,96].

In some disease models GVEC proliferation has been reported (Figure 2) [68,159]. Proliferation of GVEC does not occur without dedifferentiation of the cells [68,159], but dedifferentiation does not neccesarily lead to proliferation of GVEC [25,40]. The highly specialized structure and function resulting in terminal differentiation does not allow proliferation of the cells [151,235]. However, as with other cells, the dedifferentiated podocyte may become more sensitive to paracrine growth factors produced in renal diseases such as bFGF [214], EGF [5,50,52,216] or TGF-ß [139,162], and proliferate. Whether these growth factors play a role in the accumulation of epithelial cells as reported in glomerular crescent formation [101] is at present unknown.

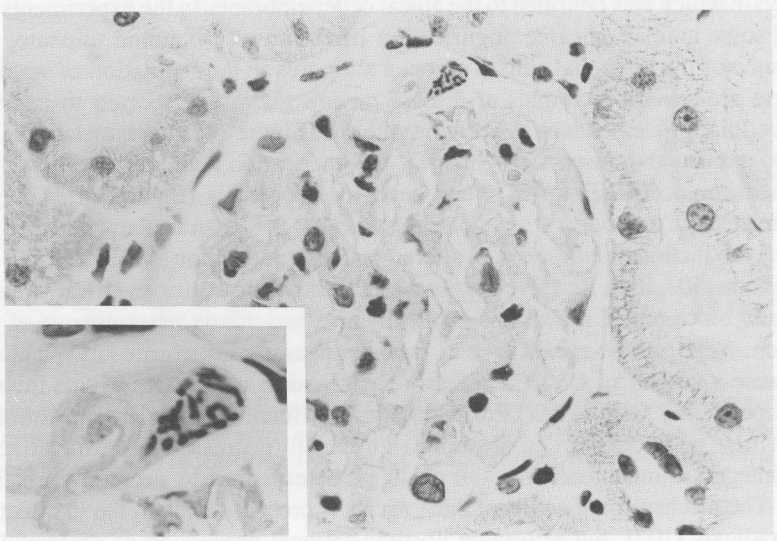

Figure 2: Incidental podocyte mitosis as observed in a plastic section (HE; Technovit-8100; Kulzer GmbH, Germany) of a kidney of a DZB rat with mercury-induced membranous nephropathy (see chapter 5). x560. Inset: detail x1400.

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Active role of podocytes in glomerular inflammation

There are several arguments to ascribe an important role to the podocyte in humoral and cellular aspects of glomerular inflammation. GVEC are capable of regulating complement activation by binding of complement-factors by specific receptors, production of complement-factors and production of inhibitors of complement-activation (Table 1). Podocytes can bind (auto) antibodies through specific Fc-receptors [144] and even resolve subepithelial immune complexes by Fc-receptor mediated endocytosis [7,203]. GVEC can produce oxygen radicals [164] and thus directly damage for example the GBM and activate inflammatory processes, but they can also inhibit this pathway by production of scavengers [245]. The podocytes can interfere with platelet aggregation and fibrinolysis by production of ADP [178] and u-PA and t-PA respectively [88,100]. GVEC are able to respond to cytokines such as IFN-γ and IFN-α by targeted cell-membrane expression of MHC class II and ICAM-1 (chapter 6). The cytokines IL-4 and IFN-γ only disturb monolayer integrity of cultured GVEC when administered to the basolateral cell surface, again demonstrating specific (receptor mediated) responsiveness (chapter 5). Production of cytokines by GVEC has not been reported yet, but biochemically they resemble other epithelial cells which produce cytokines [11,238]. Podocytes can be actively involved in cell-mediated immunity by expressing adhesion molecules such as ICAM-1 (chapter 6) thus facilitating a prolonged stay of infiltrated inflammatory cells in the glomerulus. In addition, podocytes can process and present antigens in the context of MHC class II [146] to inflammatory cells (chapter 6). GVEC may even initiate inflammatory cell influx by production of an array of chemotactic factors (Table 1). This may well explain the role of podocytes in the development of glomerular sclerosis as recently reviewed by Wolthuis et al. [242].

Conclusion and future prospects

It has become clear that podocytes have important functions *in vivo*, not only in the regulation of water flow, but also in communication with other renal parenchymal and inflammatory cells, thus contributing to the establishment of a constant glomerular microenvironment. In order to improve our understanding of these functions and to unravel the mechanisms involved, cell culture experiments will remain essential. However, the dedifferentiated morphology and physiology of cultured glomerular visceral epithelial cells are an important stumble-block for advances in this area, in particular for the study of dedifferentiation *in vivo*. The results with pressurized cell-cultures as presented in chapter 3 suggest that the use of advanced cell-culture methods together with the identification of crucial growth factors or supplements may help to tackle this problem. Probably the most important parameter for induction of differentiation *in vitro* (and *in vivo*) is the expression of negatively charged cell surface molecules such as podocalyxin. It may be worthwhile to develop stably transfected cell-lines that constitutively express high levels of these molecules. By inhibition of proliferation, application of basolateral hydrostatic pressure and expression of negatively charged cell surface molecules it may then be possible to achieve ultimate differentiation of podocytes *in vitro*, which will allow more accurate and more reliable studies to the structure and function of podocytes.