original article

Direct effects of dexamethasone on human podocytes

C-Y Xing[1,2], MA Saleem[1], RJ Coward[1], L Ni[1], IR Witherden[1] and PW Mathieson[1]

[1]Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol, UK and [2]The First Affiliated Hospital, Nanjing Medical University, Nanjing, People's Republic of China

Glucocorticoids are widely used in the treatment of human glomerular diseases, but their mode of action is poorly understood particularly in steroid-sensitive nephrotic syndrome, which is most common in childhood and is characterized by a lack of inflammation in the kidney. The podocyte is a key cell in the glomerulus in health and disease: until recently, human podocytes have been difficult to study _in vitro_. We have developed a conditionally immortalized human podocyte cell line transfected with a temperature-sensitive simian virus 40 transgene: when the transgene is inactivated _in vitro_, these cells adopt the phenotype of differentiated podocytes. We have used these cells to evaluate, using immunocytochemistry, reverse transcriptase-polymerase chain reaction, and Western blotting, direct effects of the glucocorticoid dexamethasone at concentrations designed to mimic _in vivo_ therapeutic corticosteroid levels. Dexamethasone upregulated expression of nephrin and tubulin-α, and downregulated vascular endothelial growth factor. Effects on cell cycle were complex with downregulation of cyclin kinase inhibitor p21 and augmentation of podocyte survival, without any effect on apoptosis. We report cytokine production by human podocytes, especially interleukin (IL)-6 and -8; IL-6 expression was suppressed by dexamethasone. These potent direct effects on podocytes illustrate a novel mode of action of glucocorticoids and suggest potential new therapeutic strategies for glomerular disease.

Glucocorticoids exert potent effects on leukocytes and cells of the immune system, forming the mainstay of treatment for many human inflammatory diseases. Their effectiveness in diseases where there is no evident inflammation is more difficult to understand: one conspicuous example is minimal change nephropathy, a major cause of nephrotic syndrome especially in children. Nephrotic syndrome is characterized by massive leakage of protein into the urine: the disease and its treatment cause major morbidity and mortality.[1] For decades, it has been assumed that immunological disturbances underlie this condition, with effectiveness of glucocorticoids being cited in the seminal paper by Shalhoub[2] as one of the best lines of evidence for this assumption. Recent evidence from knockout mice and rare single human gene disorders emphasizes the importance of podocytes (visceral glomerular epithelial cells) in nephrotic syndrome: defects of the podocyte-specific gene nephrin[3,4] cause congenital nephrotic syndrome; mutations in another podocyte-specific gene podocin[5] also cause severe early-onset nephrotic syndrome. Deletion in mice of CD2-associated protein, which associates with nephrin,[6] causes congenital nephrotic syndrome.[7] The limited ability of podocytes to replicate and/or repair may underlie the tendency for glomerular diseases to progress to scarring (glomerulosclerosis).[8,9] We tested the hypothesis that glucocorticoids exert direct effects on podocytes. Previously, the only available human podocytes were transformed cell lines or undifferentiated primary culture cells: these cells are not considered representative of mature podocytes. We developed conditionally immortalized human podocytes using a temperature-sensitive transgene: when cells are moved to the non-permissive temperature of 37°C, the transgene is silenced and cells develop a mature podocyte phenotype.[10] We now report potent direct effects of the glucocorticoid dexamethasone on these cells, at concentrations designed to mimic _in vivo_ therapeutic corticosteroid levels. Our results illustrate a novel mode of action of glucocorticoids, suggesting that efficacy of these drugs in nephrotic syndrome may not be explained by conventional anti-inflammatory or immunosuppressive actions. The data add to evidence that the podocyte is the key cell in human glomerular disease and suggest novel therapeutic strategies.

Correspondence: _PW Mathieson, Academic Renal Unit, University of Bristol, Southmead Hospital, Bristol BS10 5NB, UK. E-mail: p.mathieson@bris.ac.uk_

RESULTS

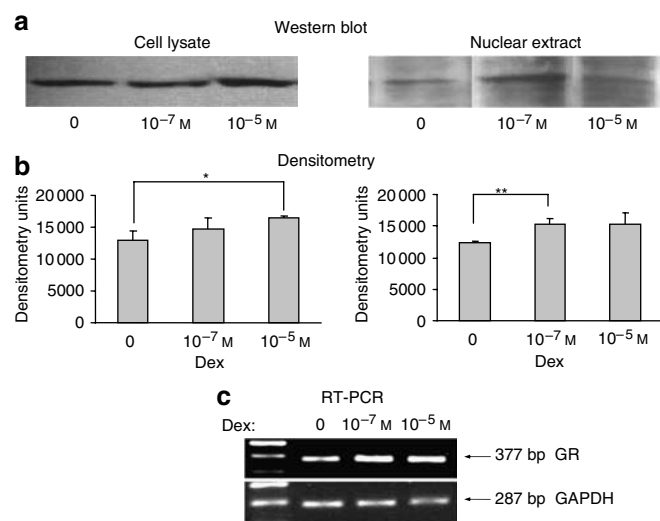### Expression of glucocorticoid receptors by podocytes

First, we confirmed that expression of glucocorticoid receptors by podocytes, as previously reported in human kidney sections,[11] was replicated by our podocyte cell line *in vitro*. Glucocorticoid receptors were present in both nuclear and cytoplasmic extracts (Figure 1). There was a suggestion that overall level of expression and nuclear localization of glucocorticoid receptors, quantitated by Western blotting in whole-cell lysates and in nuclear extracts, was upregulated by dexamethasone in a dose-dependent manner, although this only achieved statistical significance in cell lysates with 10^{-5} M dexamethasone and in nuclear extracts with 10^{-7} M dexamethasone (Figure 1).

Effects of dexamethasone on podocyte maturation at 37°C and expression of nephrin and tubulin α
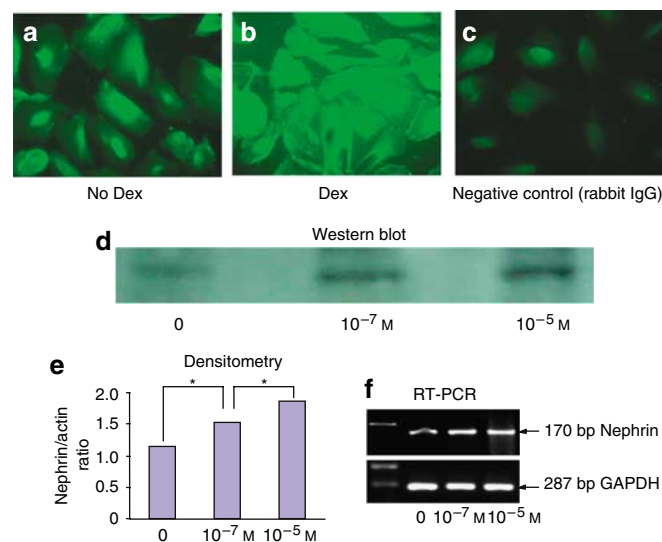
When these conditionally transformed cells are cultured at the permissive temperature of 33°C, the transgene is active and cells proliferate into a cobblestone epithelial monolayer similar to that seen with primary culture cells.[10] When moved to non-permissive temperature of 37°C, the transgene is silenced, cells mature and form complex processes, assuming the phenotype of mature podocytes.[10] Maturation was enhanced and accelerated by dexamethasone: processes started to form at days 12 and 13 without dexamethasone, at days 10 and 11 with 10^{-7} M dexamethasone, and at days 7 and 8 with 10^{-5} M dexamethasone.

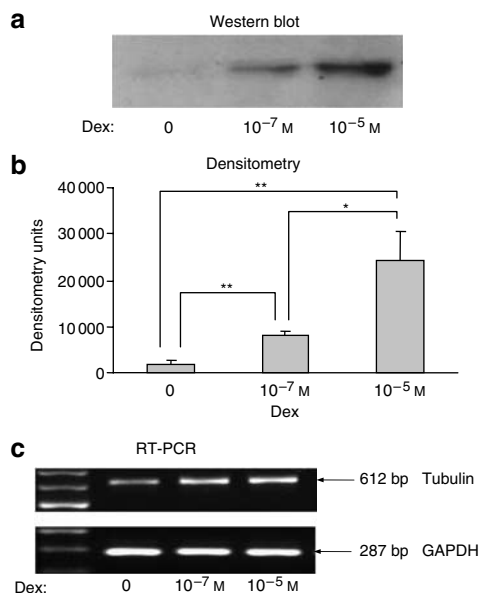Nephrin expression was upregulated after 14 days incubation with dexamethasone (Figure 2). Immunofluorescent staining is not regarded as a quantitative technique, but there is a striking difference in the amount of fluorescence seen in images taken on identical exposure settings with anti-nephrin rabbit polyclonal antibody K2966 according to the presence or absence of dexamethasone in the culture medium for 14 days (Figure 2a, no dexamethasone, Figure 2b, cells incubated with 10^{-5} M, Figure 2c negative control immunofluorescence with rabbit immunoglobulin (Ig) for comparison). Quantitation by Western blot with rabbit polyclonal anti-nephrin antibody K2737 confirmed dose-dependent upregulation of nephrin protein by dexamethasone (Figure 2d, representative of five replicate experiments). Nephrin is a large-molecular-weight membrane-bound protein and is known to be difficult to blot, accounting for the indistinct bands. We therefore show detailed densitometric quantification of three replicate Western blots, which showed nephrin/actin ratios of 1.0:1.33:1.618 with 0, 10^{-7}, and 10^{-5} M dexamethasone, respectively ($P < 0.05$, Figure 2e). Reverse transcriptase-polymerase chain reaction (RT-PCR) suggested that this effect was at least partly at the RNA level (Figure 2f, representative of five replicate experiments). Expression of other key podocyte proteins, including podocin,[5]

Figure 2 | **Effects of dexamethasone on the expression of nephrin in human podocytes.** (**a–c**) Effect of incubation with dexamethasone on expression of nephrin in human podocytes by immunofluorescence with rabbit polyclonal antibody K2966 (all pictures taken at identical exposure settings, green staining indicates nephrin protein present). (**a**) Incubation in standard culture medium, without dexamethasone for 14 days with medium changed on alternate days. (**b**) Incubation with standard medium plus 10^{-5} M dexamethasone for 14 days with medium changed on alternate days. (**c**) Negative control immunofluorescence on podocytes incubated with standard medium plus 10^{-5} M dexamethasone for 14 days with medium changed on alternate days, using rabbit Ig instead of primary antibody. (**d**) Western blot on podocyte whole-cell lysate using rabbit polyclonal anti-nephrin antibody K2737 (representative of five replicate experiments). (**e**) Densitometric quantification of three replicate Western blots, expressed as nephrin/actin ratio (mean ± s.e.m.). Mean ratio for $0 : 10^{-7} : 10^{-5}$ M = 1:1.33:1.618 (*$P < 0.05$). (**f**) RT-PCR on podocyte RNA using primers for nephrin and 'housekeeping' gene GAPDH (representative of five replicate experiments).

Figure 1 | **Effects of dexamethasone on expression of glucocorticoid receptors in human podocytes.** (**a**) Representative Western blot on whole-cell lysates and nuclear extracts. (**b**) Densitometry of five replicate Western blots for cell lysates, and three replicate Western blots for nuclear extracts (*$P < 0.05$, **$P < 0.01$). (**c**) RT-PCR on podocyte RNA using primers for glucocorticoid receptor (GR) and control 'housekeeping' gene (GAPDH) (representative of three replicate experiments).
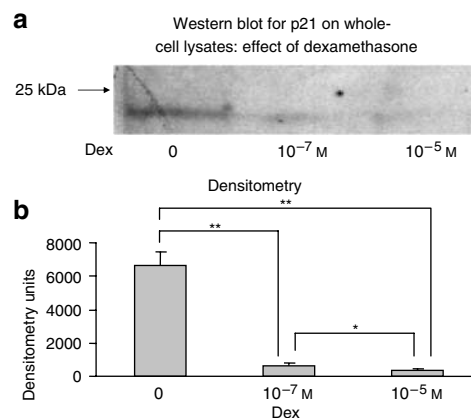
Figure 3 | Effects of dexamethasone on the expression of tubulin α in human podocytes. (**a**) Western blot on whole-cell lysate (representative of three replicate experiments). (**b**) Densitometry of three replicate Western blots (*$P < 0.05$, **$P < 0.01$). (**c**) RT-PCR on podocyte RNA using primers for tubulin α and 'housekeeping' gene GAPDH (representative of three replicate experiments).

Figure 4 | Effects of dexamethasone on the expression of cyclin kinase inhibitor p21 in human podocytes. (**a**) Western blot on whole-cell lysates (representative of three replicate experiments). (**b**) Densitometry of three replicate Western blots (*$P < 0.05$, **$P < 0.01$).

CD2-associated protein,[7] and synaptopodin[10] was unaffected by dexamethasone (data not shown).

Enhanced podocyte maturation and formation of major processes with dexamethasone was accompanied by increased expression of microtubule protein tubulin α, quantitated by densitometry of Western blots (Figure 3a and b). RT-PCR (Figure 3c, representative of three replicate experiments) suggested that upregulation was at least partly at the RNA level.

Effects of dexamethasone on apoptosis and podocyte number

In many cell types, dexamethasone induces apoptosis.[12,13] However, dexamethasone had no effect on apoptosis in podocytes: percentage apoptotic cells, respectively, with 0, 10^{-7}, and 10^{-5} M dexamethasone were 3.7, 3.0, and 3.7 at 33°C, and 4.7, 4.3, and 6.0 at 37°C ($P > 0.05$). Despite this lack of effect on apoptosis, dexamethasone significantly enhanced podocyte number. Under permissive (33°C) conditions, cell index (ratio of number of cells compared to baseline) was 2.100 at day 7. This was increased to 3.537 by 10^{-7} M dexamethasone ($P < 0.01$) and to 2.639 by 10^{-5} M dexamethasone ($P < 0.05$). We speculated that this enhancement of podocyte number was owing to increased survival of individual cells, and tested this using long-term podocyte culture. When cultured for 60 days at 37°C without dexamethasone, 34.6% of podocytes survived. At a wide range of dexamethasone concentrations, podocyte survival was enhanced, percentage survival at day 60 being 75.4, 80.6, 67.5, 69.2, and 87.6 respectively, at day 60 with 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M dexamethasone.

**Effects of dexamethasone on p21 in differentiated podocytes**

Expression of cyclin kinase inhibitor p21 was assessed by immunocytochemistry and Western blotting in podocytes. Without dexamethasone, all cells were p21 positive, although intensity of p21 staining was variable. Dexamethasone markedly reduced intensity of staining and percentage of cells staining positive. The effects were quantified by Western blot on whole-cell lysates (Figure 4), confirming downregulation of p21 protein.

**Effects of dexamethasone on transcription factor expression**

We studied transcription factor GATA-3 because of its possible role in the regulation of nephrin expression: the nephrin promoter contains multiple putative GATA-3 binding domains.[14] By immunocytochemistry, all podocyte nuclei expressed GATA-3. Dexamethasone caused marked downregulation of GATA-3 protein quantified by Western blotting and GATA-3 RNA on RT-PCR (Figure 5). Other transcription factors analyzed were nuclear factor (NF)-κB and NF-1: both were abundantly expressed by podocytes but unaffected by dexamethasone (data not shown).

Effects of dexamethasone on VEGF expression

Expression of vascular endothelial growth factor (VEGF) was analyzed by immunocytochemistry, Western blotting, and RT-PCR (Figure 6). VEGF is differentially spliced in podocytes;[15,16] available antibodies do not discriminate between isoforms so that isoform expression pattern can only be assessed at mRNA level. In differentiated podocytes, VEGF was expressed in a linear distribution in cytoplasm and in cell processes. Dexamethasone downregulated VEGF expression; with 10^{-5} M dexamethasone, VEGF expression was virtually absent by immunofluorescence. Quantitative effects were confirmed by Western blotting (Figure 6a and b, representative of three replicate experiments, $P < 0.01$). RT-PCR showed that podocytes expressed mRNA for at least three VEGF isoforms (VEGF121, −165, −189).
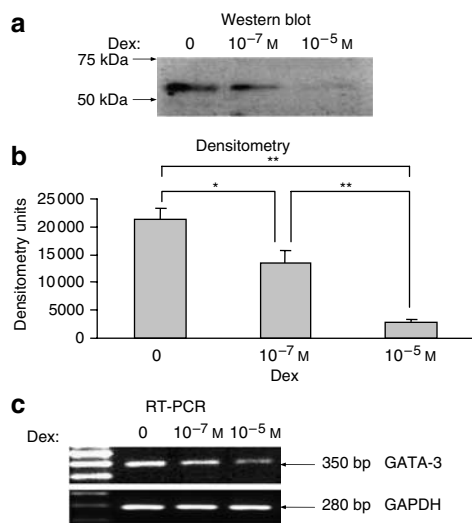
Figure 5 | Effects of dexamethasone on the expression of transcription factor GATA-3 in human podocytes. (a) Western blot on nuclear extract (representative of three replicate experiments, $P < 0.01$) (b) Densitometry of three replicate Western blots (*$P < 0.05$, **$P < 0.01$). (c) RT-PCR on podocyte RNA for GATA 3 and 'housekeeping' gene GAPDH (representative of three replicate experiments).
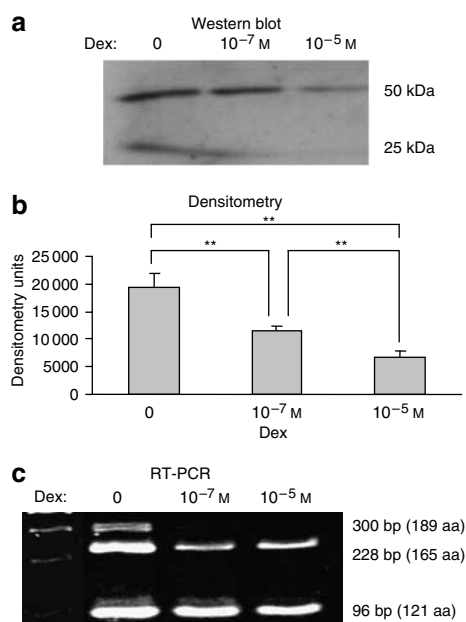
Figure 6 | Effects of dexamethasone on the expression of VEGF in human podocytes. (a) Western blot on whole-cell lysate (representative of three replicate experiments). (b) Densitometry of three replicate Western blots (*$P < 0.05$, **$P < 0.01$). (c) RT-PCR on podocyte RNA:VEGF primers amplify multiple isoforms of VEGF (representative of three replicate experiments).

Dexamethasone downregulated all three isoforms, but particularly VEGF165 and −189 (Figure 6c, representative of three replicate experiments).

Cytokine expression by podocytes; effects of dexamethasone

Supernatants of cultured mature human podocytes contained pg/ml quantities of interleukins (IL)-6 and -8 but no detectable IL-1β, IL-2, IL-4, IL-10, IL-12, tumor necrosis factor-α, or interferon-γ. The effect of dexamethasone treatment of mature cells for 48 h was tested because cytokine expression was expected to be rapid and maximal within this time period. Furthermore, the short half-life of secreted cytokines made it important to test at early time periods. Dexamethasone suppressed IL-6 production (fluorescence intensity in cytokine bead array, mean $\pm$ s.d., 1502 ± 619 without Dex to 878 ± 342 after 48 h with 10^{-5} M Dex, $n = 9$, two-tailed $P = 0.0039$, Wilcoxon non-parametric paired analysis) but had no effect on IL8.

DISCUSSION

Nephrotic syndrome, especially in childhood, often responds to treatment with corticosteroids: this is unlikely to be owing to conventional anti-inflammatory effects of these drugs since steroid-sensitive nephrotic syndrome is typically associated with complete absence of glomerular inflammation. Other possible mechanisms of the beneficial effect of corticosteroids could include direct protection of podocytes from injury and/or promotion of podocyte repair. We were interested to test the hypothesis that the typical glucocorticoid dexamethasone has direct effects on human podocytes. Such studies have previously been hampered by lack of suitable cell lines: we have developed conditionally transformed human podocytes, which at 37°C differentiate into a phenotype closely resembling mature podocytes.[10] We found that dexamethasone has potent direct effects on human podocytes in vitro.

The podocyte is a highly specialized and complicated cell that plays a critical role in the kidney in health and disease.[17–19] Recent advances have highlighted the importance of podocytes in nephrotic syndrome, where the glomerular filtration barrier is disrupted and there is massive leakage of protein into urine.[19,20] The podocyte-specific gene nephrin seems particularly important: mutations of this gene in man cause congenital nephrotic syndrome of Finnish type[3] and nephrin-deficient mice also develop congenital nephrotic syndrome.[4] Regarding acquired forms of nephrotic syndrome in man, an early report suggested that nephrin expression was reduced.[21] This remains controversial, soon being confirmed by one report[22] but refuted by another.[23] However, there is general agreement that the podocyte is an important target of injury in many forms of acquired nephrotic syndrome.[20,24,25] As mentioned earlier, defective repair mechanisms in podocytes may underlie the tendency for many glomerular diseases to lead to progressive scarring culminating in kidney failure.[8,9] In particular, it is believed that the limited capacity of podocytes to replicate hampers their ability to repair themselves and/or replace injured podocytes with new progeny.[26] In health, podocytes are restrained from entry into cell cycle by a complex array of inhibitory molecules.[26] In diseases characterized by podocyte injury, including glomerulonephritis and diabetic nephropathy, normal regulation of these inhibitors is disturbed and in particular the cyclin kinase inhibitor p21 is upregulated.[26–28] Our data

showing that dexamethasone leads to marked downregulation of p21 expression may therefore be particularly significant: podocyte proliferation and repair may require the 'molecular brake' to be taken off and release of cell cycle inhibition by downregulation of p21 could allow this to occur.

Concentrations of dexamethasone were based on calculations of 'therapeutic' concentrations *in vivo*. Loew *et al.*[29] found peak plasma dexamethasone concentrations of $0.88 \pm 0.15 \times 10^{-7}$ M in normal humans after intramuscular injection of 3 mg dexamethasone. After intravenous injection of 1.0 g methylprednisolone, dexamethasone-equivalent peak concentration (adjusted for glucocorticoid potency) was $2.95–8.49 \times 10^{-6}$ M.[30] Patients with nephrotic syndrome receiving high-dose oral or intravenous prednisolone had peak concentrations of total and unbound prednisolone in plasma of about 450 and 173 μg/ml, respectively.[31] Equivalent dexamethasone concentrations, incorporating a correction factor for glucocorticoid potency, are 1.72×10^{-7} and 0.66×10^{-7} M, respectively. To be consistent with clinical doses of glucocorticoid used in the treatment of glomerular diseases, we therefore chose for our quantitative analyses, the concentrations of dexamethasone of 1×10^{-7} and 1×10^{-5} M.

Glucocorticoids exert cellular effects by binding to glucocorticoid receptors in cytoplasm, forming dimers and being translocated into the nucleus where they bind to glucocorticoid response elements in DNA, or interact with other transcription factors, thus regulating transcription of many genes.[32] Glucocorticoid receptors are known to be expressed by podocytes in human kidney,[11] and we have found that human podocytes *in vitro* also express glucocorticoid receptors in the expected nuclear and cytoplasmic distribution; dexamethasone caused modest upregulation and increased nuclear localization of glucocorticoid receptors. In other cell-types, glucocorticoids have been shown to have varied effects on glucocorticoid receptor expression.[33]

We found that dexamethasone enhanced and accelerated podocyte maturation, with a particularly striking effect on expression of nephrin. Nephrin is a key component of the slit diaphragm, the main site of control of glomerular permeability. Our data suggest that dexamethasone-induced upregulation of nephrin occurred at least partly at the RNA level. This interpretation must be cautious however: simple RT-PCR is not reliable for quantitation and future studies should use Northern blotting and/or fully quantitative PCR. Our finding that GATA-3 was downregulated does not support the suggestion that GATA-3 is important in nephrin gene transcription. Podocin, CD2-associated protein, and synaptopodin were not affected by dexamethasone.

Dexamethasone enhanced podocyte survival without any effect on apoptosis. In other cell types, glucocorticoids have variable effects on proliferation, differentiation, and apoptosis; clearly, there are cell-type-specific effects.[12,13] In human and experimental studies, it has been suggested that the inability of the mature podocyte to undergo mitosis is responsible for the development of glomerulosclerosis and progression of glomerular disease.[8,9] However, in some forms of glomerular disease including HIV-associated nephropathy, unregulated podocyte proliferation does occur in response to injury, and is considered deleterious.[34] Clearly, novel forms of therapy aimed at allowing podocyte repair would need to be carefully targeted to prevent excessive podocyte proliferation. Our data suggest that p21 may be a particularly important target.

VEGF is a mitogen for vascular endothelial cells, plays an important role in vasculogenesis and angiogenesis, and induces vascular leakage and vasodilation.[15] In normal glomeruli, VEGF is expressed exclusively by podocytes[35] and is differentially spliced into isoforms (VEGF121, −148, −165, −189), which are thought to differ in tissue-binding characteristics and functions.[15,16] Upregulation of VEGF in renal tissue from patients with minimal change nephropathy compared to controls and other glomerular disease has been reported,[36,37] so an effect of glucocorticoids to downregulate VEGF expression may be beneficial.

Cytokine production by podocytes may provide the previously elusive link[38] between the podocyte and the immune system. Local production of IL-6 by podocytes may contribute to the pathogenesis of nephrotic syndrome, so that its suppression by corticosteroids is intriguing.

Putting our observations together, we propose the following mechanistic hypothesis. In nephrotic syndrome, podocytes lose the complex processes that characterize their differentiated state. Expression of nephrin is disrupted. Corticosteroids act directly on podocytes to promote repair, with enhanced process formation and upregulation of nephrin. We speculate that the downregulation of p21, which normally acts as a molecular 'brake' on podocytes' ability to enter the cell cycle,[26,27] is important in allowing this to occur. Thus, the podocyte's limited ability to repair itself, thought to lie behind the importance of this cell type in progressive glomerular injury,[9] can be overcome by exogenous corticosteroid. Novel approaches to therapy for nephrotic syndrome should concentrate on measures aimed at enhancing podocyte repair and nephrin expression. Our cell line will provide a valuable tool for assessment of these approaches. Consistent with these ideas are our recent observations[39] that incubation with plasma from patients with active nephrotic syndrome, but not from the same patients in remission, induces marked phenotypic changes in human podocytes with loss of process formation and downregulation of nephrin expression, that is, opposite changes to those seen after treatment of the cells with dexamethasone. In mouse podocytes, a recent proteomic analysis reported effects of dexamethasone on numerous genes.[40] A possible functional relevance of these observations is illustrated by a previous report that dexamethasone protected mouse podocytes *in vitro* from the permeability-increasing effects of adriamycin.[41] Thus, evidence is accumulating to support the importance of corticosteroids' effects on podocytes as an

explanation for their beneficial therapeutic effects in glomerular disease.

In summary, the glucocorticoid dexamethasone has direct effects on human podocytes through regulating glucocorticoid receptors, cell maturation and survival, cytoskeleton, and expression of key proteins nephrin, VEGF, and IL-6. The direction of these changes would be beneficial in reversing pathological changes observed in steroid-sensitive nephrotic syndrome. Together with recent observations that emphasize the primary role of the podocyte in many forms of glomerular disease, this leads us to suggest that the effects of glucocorticoids in nephrotic syndrome may have little to do with conventional anti-inflammatory or immunosuppressive actions of these drugs, instead reflecting modulation of podocyte biology and promotion of podocyte repair mechanisms.

MATERIALS AND METHODS
Podocyte culture
Conditionally immortalized human podocytes were derived and cultured as reported:[10] from a discarded nephrectomy specimen from a 3-year-old child whose parents gave full informed consent. Passage numbers of cells were between 5 and 18, batches of cells of same passage number being used for each set of experiments. Replicate experiments were performed (number of experiments is stated in each case below) and representative results are shown. Medium, with or without added dexamethasone as appropriate, was changed on alternate days throughout.

Treatment with dexamethasone
Dexamethasone-water soluble (Sigma-Aldrich Co., Poole, UK) was dissolved in culture medium at concentrations ranging from 10^{-8} to 10^{-2} M; fresh dexamethasone-containing medium or control medium was supplied on alternate days. As control in each study, cells were cultivated contemporaneously for the same number of days in culture medium without dexamethasone. Unless otherwise stated, results shown are after 14 days treatment with dexamethasone or control. Detailed quantitative assessments were mainly based on the comparison between 0, 10^{-7}, and 10^{-5} M dexamethasone, as these latter two concentrations equate to 'therapeutic' concentrations achieved in vivo (see, Discussion).

Antibodies and control Igs
Primary antibodies were as follows: mouse monoclonal and rabbit polyclonal anti-nephrin (kind gifts of Dr Karl Tryggvason); rabbit polyclonal anti-podocin (kind gift of Dr Corinne Antignac); and mouse monoclonals anti-tubulin α (Zymed Laboratories, South San Francisco, CA, USA), anti-p21, anti-GATA-3, rabbit polyclonals anti-glucocorticoid receptor, anti-VEGF (all Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies were: fluorescein (fluorescein isothiocyanate)-conjugated affinity-purified goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Controls were as follows: mouse-IgG1 (Sigma-Aldrich Co.), mouse-IgG, rabbit-IgG, and goat-IgG (Santa Cruz).

Cell number – MTS assay
Cell number was measured using MTS (Promega, Southampton, UK), which is bioreduced into a soluble formazan that can be quantitated by spectrophotometry and is directly proportional to the number of living cells. Standard curves were obtained using known numbers of cells.

Apoptosis – TUNEL assay
Apoptosis was measured by terminal deoxynucleotide transferase (TdT)-mediated dUTP-fluorescein isothiocyanate nick-end labeling (TUNEL) assay (Promega kit).

Immunofluorescence
Cells were grown on collagen type I-coated glass coverslips, fixed with paraformaldehyde, and then permeabilized with Triton X-100. Nonspecific binding was blocked with 3% bovine serum albumin plus species-control IgG. Primary antibodies were applied at one in 100 dilution in blocking buffer for 60 min at room temperature. After washing, cells were incubated with secondary antibodies diluted one in 100 in blocking buffer. Results were visualized by immunofluorescence microscopy, photographed with a digital camera.

RT-PCR
Total RNA was extracted by phenol–chloroform guanidine thiocyanate (Promega). Total RNA (0.5–1.0 μg) was reverse transcribed using Promega's Reverse Transcription System. Table 1 shows PCR primers, predicted product sizes, and amplification conditions. cDNA (0.5–3 μl) was added to final volume 25 μl, containing 10 pM

Table 1 | Primers and PCR conditions

Name	Forward primer	Reverse primer	Product size (bp)	Annealing temperature (°C)	Cycles
Nephrin	5′-GAC CGA GTC AGG AAC GAA TA-3′	5′-CCT GTG AAA CCT CGG GAA TA-3′	170	58	40
Tubulin α	5′-TCG CAA GCT GGC TGA CCA GT-3′	5′-TTG GGA ACC ACG TCA CCA CG-3′	612	64	30
VEGF Isoforms	5′-GTG AAT GCA GAC-3′ 5′-CAA AGA AAG-3′	5′-AAA CCC TGA GGG-3′ 5′-AGG CTC-3′	96, 228, 300, 351	58	40
GATA-3	5′-AGG TAC CCT CCG ACC CAC CA-3′	5′-CTC AAG TAC CAG GTG CCC CT-3′	350	55	35
GAPDH	5′-GTA GAG GCA GGG-3′ 5′-ATG ATG TT-3′	5′-GCT GTA GGA AGC-3′ 5′-TCATCTCT-3′	287	56	30

GAPDH, glyceraldehyde-3-phosphate; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor.

of each primer, 1–2.5 U of *Taq* DNA polymerase, 1.5–2.5 mM MgCl$_2$, and 100 μM of dNTPs. Products were electrophoresed in 2% agarose and visualized by ethidium bromide staining. In semiquantitative experiments, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified for comparison.

Western blot

Total cell lysates were prepared from podocytes using radio-immunoprecipitation assay buffer (Sigma-Aldrich). Nuclear extracts were prepared using Nuclear™ Extraction Kit (Sigma-Aldrich). Proteins were separated by 8% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Sigma-Aldrich). After blocking with bovine serum albumin plus dry milk (Sigma-Aldrich) and control IgG, membranes were incubated sequentially with primary antibodies diluted one in 500 in blocking buffer, horseradish peroxidase-conjugated secondary antibodies diluted one in 10 000 (for monoclonal primary) or one in 20 000 (for polyclonal primary), followed by Chemiluminescence Luminol Reagent (Santa Cruz). X-ray film was exposed to membrane, developed, and scanned. In all experiments, protein loading was standardized by preliminary experiments in which actin expression was quantitated. Blots were then probed for protein of interest band intensity assessed by densitometry.

Densitometry

Gels were scanned by densitometry and analyzed by Labworks 4.0 (UVP Inc., Upland, CA, USA).

Cytokine expression

Culture supernatants were analyzed using cytokine bead array (Becton Dickinson, Oxford, UK), which allows simultaneous measurement of multiple cytokines: IL-1β, -2, -4, -6, -8, -10, and -12, tumor necrosis factor-α, and interferon-γ. Supernatants were incubated with beads for 1 h at room temperature then analyzed by flow cytometry. Manufacturer's standards allow quantitation of each cytokine, the fluorescence intensity with each bead size being proportional to the concentration of individual cytokines.

Statistical analysis

Statistical analysis was performed using SPSS10 software. Pooled results from replicate quantitative experiments were compared by analysis of variance. Two-tailed *P*-values of less than 0.05 were taken as indicating statistical significance.

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