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Distinct Pathogenic Effects of Group B Coxsackieviruses on Human Glomerular and Tubular Kidney Cells

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The six group B coxsackieviruses (CVBs) are highly prevalent human pathogens that cause viremia followed by involvement of different organs. Clinical and experimental evidence suggests that CVBs can induce kidney injury, but the susceptibility of human renal cells to these viruses is unknown. By using pure cultures of human glomerular and tubular cells, we demonstrated that all CVBs are capable of productively infecting renal cells of three different histotypes. Distinct pathogenic effects were observed. Proximal tubular epithelial cells and, to a lesser extent, glomerular podocytes were highly susceptible to CVBs; in both cases, infection led to cytolysis. In contrast, glomerular mesangial cells supported the replication of the six CVBs but failed to develop overt cytopathologic changes. Mesangial cells continued to produce infectious progeny for numerous serial subcultures (i.e., more than 50 days), especially with type 1, 3, 4, and 5 viruses. In the above cells, persistent infection induced the de novo synthesis of platelet-derived growth factor A/B and enhanced the release of transforming growth factor β1/2. These two factors are important mediators of progression from glomerular inflammation to glomerulosclerosis. CVB replication appeared also to impair the phagocytic and contractile activity of mesangial cells. Loss of these properties—which are important in glomerular physiopathology—may contribute to the development of progressive nephropathy. The results show that CVBs induce distinct effects in different types of cultured renal cells and suggest that CVB infections may be associated with both acute and progressive renal injury.

Viruses may be the cause of renal diseases (31, 70). Although the importance of the kidneys in virus persistence and transmission has been appreciated for a long time, only a few studies have tried to clarify the underlying mechanisms. It is usually held that renal injury associated with acute viral infections is minimal and transient and that serious virus-induced nephropathy is the result of postacute immune system-mediated reactions rather than of local virus replication (26). Nephrotropic agents such as polyomaviruses, mumps virus, and cytomegalovirus (CMV) are shed into urine after replication in the epithelial cells of renal tubuli (35, 54, 55, 62, 72), but the precise site of virus replication is unknown. Knowledge on the interaction of viral agents with glomerular cells is especially scant: glomerular mesangial cells appear to allow the replication of CMV (28) and Puumala virus (68), a bunyavirus recognized as the etiologic agent of nephropathia epidemica. Glomerular and tubulointerstitial alterations are a common finding in human immunodeficiency virus type 1 (HIV-1) infection, but results on the susceptibility of cultured renal cells to HIV are controversial (1, 36). Glomerulonephritis is frequently observed as a manifestation of immune complex disease, for instance in chronic hepatitis virus B infection (66). Clinical and epidemiological observations have also suggested that microbial antigens are implicated in the pathogenesis of immunoglobulin A (IgA) nephropathy (23) and that some cases of "idiopathic" glomerulonephritis are caused by unidentified infectious agents (70).

The six group B coxsackieviruses (CVBs) are human pathogens that may cause either asymptomatic infection or serious diseases such as cardiomyopathy, meningitis, myositis, and possibly insulin-dependent diabetes (45, 56). CVB infections are characterized by primary viral replication at the portal of entry followed by viremia that, in turn, leads to secondary involvement of different organs. The distinct tropism of different virus strains is probably responsible for the variable clinicopathological manifestations of CVB infections. Occasional reports of acute or chronic glomerulonephritis and hemolytic uremic syndrome indicate that CVBs can induce renal disease (11, 25). CVB-4 appeared to induce mesangioproliferative glomerulonephritis in experimentally infected mice (40, 49, 50). Prolonged expression of the CVB-4 genome was detected by in situ hybridization in the glomerular mesangium of these animals (49). Despite these suggestive findings, the susceptibility of human renal cells to CVBs has not been investigated.

This study was aimed at evaluating whether primary cultures of human renal cells derived from the glomeruli or cortical tubules were susceptible to the six CVB types. Pure cultures of glomerular mesangial cells, glomerular epithelial cells ("podocytes"), and proximal tubular epithelial cells were obtained from five different donors. CVB infection was monitored by determining the titer of the virus progeny and searching for viral genomes in infected cells. Previous studies, in vivo and in vitro, have shown that CVBs can cause persistent infection in certain human tissues and that CVB persistence is often associated with long-term pathological effects (16, 38, 48). We therefore investigated if CVBs were able to persist in renal cells and if virus infection could influence the physiological functions of these targets.

MATERIALS AND METHODS

Human renal cell cultures. Primary cultures of human renal cells were obtained by the method of Striker and Striker (65) from five surgically removed

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kidneys with polar carcinomas. Unless otherwise specified, plasticware was from Falcon (Becton-Dickinson, San Jose, Calif.) whereas chemicals, tissue culture reagents, low-endotoxin-type fetal calf serum (FCS), and immunochemicals were from Sigma Chemical Co. (St. Louis, Mo.). Endotoxin levels in complete media were ≤0.01 EU/ml (*Limulus* amebocyte lysate assay; Whittaker Bioproducts, Walkersville, Md.). The lesion-free pole of each kidney was selected and decapsulated. Small cortex fragments (<2 mm^3) were suspended in RPMI 1640 and forced through a graded series of stainless steel filters (50, 100, and 200 mesh). Decapsulated glomeruli were recovered and treated with collagenase from *Clostridium histolyticum* (type D, 0.5 mg/ml for 30 min at 37°C; Boehringer, Mannheim, Germany) to remove epithelial cell components (65). Washed glomerular remnants were seeded at about 300 glomeruli/cm^2 in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Paisley, United Kingdom) containing D-valine and 20% FCS. The cultures were incubated at 37°C in 5% CO_2. D-Valine-containing DMEM was used to prevent fibroblast proliferation (47). After 2 weeks, glomerular mesangial cells (MC) were collected by digestion with trypsin-EDTA and grown in normal DMEM–20% FCS. At passage 3, cultured cells were characterized as MC on the basis of published criteria (8, 13, 19, 47): (i) stellate cells growing in interwoven bundles; (ii) uniform staining for f-actin detected with fluorescein-labeled phalloidin; (iii) staining for smooth muscle-type myosin (Immunotech, Marseilles, France); (iv) staining of the extracellular matrix with antibodies to type IV collagen and fibronectin; and (v) lack of reactivity with antibodies to HLA-DR, leukocyte common antigen (CD45; Becton-Dickinson), and human factor VIII-related antigen (FVIIIr:Ag; Nordic Immunology, Tilburg, The Netherlands). Functional criteria were also used: (i) cell contraction induced by 100 ng of angiotensin II per ml was evaluated by phase-contrast microscopy on MC seeded on glass coverslips coated with dimethylpolyxyloxane (3, 13, 61); and (ii) phagocytosis of IgG-coated latex beads was analyzed as described previously (13). The results were expressed as the percentage of MC undergoing contraction or phagocytosis.

Glomerular epithelial cells (GEC) were obtained by plating decapsulated (not collagenase-treated) glomeruli at high density in DMEM–20% FCS (21, 47, 65). After 10 to 15 days, the cultures were slightly trypsinized to remove glomerulus remnants; the outgrowing podocytes were expanded. GEC were characterized according to published criteria (2, 8, 47, 65): (i) polyhedral cells with a cobblestone-like appearance when confluent; (ii) staining for cytokeratins 8, 18, and 19 (Becton-Dickinson), vimentin, and laminin; (iii) lack of reactivity with antibodies to smooth muscle-type myosin, CD45, and FVIIIr:Ag; and (iv) cytotoxicity and cytoskeletal alterations (15) in response to puromycin aminonucleoside (10, 20, and 50 μg/ml) or adriamycin (1, 2, and 5 μg/ml).

Primary cultures of proximal tubular epithelial cells (PTEC) were obtained by plating the 200-mesh-filtered kidney cell suspension in RPMI 1640–15% FCS. After three passages, PTEC were characterized according to published criteria (7, 22, 57): (i) minimal staining for desmin and negative staining for FVIIIr:Ag; (ii) marked staining with antibodies to cytokeratins and actin; (iii) staining of >90% of cells for alkaline phosphatase as determined by the naphthol AS-MX method; and (iv) enhanced cyclic AMP production (Biotrak cyclic AMP assay system; Amersham International, Little Chalfont, United Kingdom) after stimulation with 100 nM parathyroid hormone but not with 1 mM antidiuretic hormone. Immunofluorescence of cultured cells and control experiments were performed as described previously (12).

Virus source, inoculation, and titer determination. CVB strains were originally obtained from the American Type Culture Collection (Rockville, Md.). Virus stocks were grown on KB cells, a human cell line derived from an epidermoid carcinoma. KB monolayers were infected at a low multiplicity of infection (MOI = 0.1) and incubated in serum-free RPMI 1640 for 24 h at 37°C (43). KB cultures displaying >90% cytopathic effect (CPE) were further disrupted by two freeze-thaw cycles. Cell debris was removed by centrifugation; cell-free supernatants were subjected to titer determination and stored at −70°C. The endotoxin levels in the virus preparations were <0.01 U/ml (*Limulus* assay). Virus titers were obtained by a micromethod involving KB cells and expressed as 50% tissue culture infective dose ($TCID_{50}$) per ml (43). To quantitate CVBs as PFU, serial virus dilutions were added in duplicate to KB cell monolayers (0.2 ml of virus in 60-mm culture dishes). After 30 min at 37°C, the excess liquid was removed and 5 ml of medium (DMEM–10% FCS, 20 mM HEPES) containing molten 0.75% agar (Noble agar; Difco, Milan, Italy) was added and allowed to solidify. After a 3-day incubation, viable cells were stained with neutral red (1:10,000 in DMEM; 1 ml per plate). Plaques were counted 3 and 5 days postinfection (p.i.), and the titer was expressed as PFU per milliliter.

From the fourth passage onward, primary renal cell cultures were infected with CVBs. MC, GEC, and PTEC were subcultured at 50% confluence, and 2 days later, the monolayers were washed with warm phosphate-buffered saline containing Ca^{2+} and Mg^{2+} and challenged in duplicate with the six CVB serotypes (MOI = 5) in serum-free medium. After a 1-h incubation at 37°C, the cultures were washed and supplemented with complete medium. A sample of supernatant was taken as the initial point for the time course of virus and cytokine production. CVB infection was monitored by evaluating (i) the development of CPE, (ii) extracellular virus titers, and (iii) viral genomes in infected cells.

CVB receptor expression by renal cells. Since different CVB types appear to share the major surface receptors (6, 14, 69), the expression of CVB membrane receptors on MC and PTEC was evaluated by measuring the kinetics of CVB-3 attachment to cultured cells. MC and PTEC were detached with 1 mM PBS-EDTA, divided into aliquots (10^6 cells in 0.1 ml of serum-free DMEM), and incubated at 37°C with 0.1 ml of CVB-3 (10^6 PFU; MOI = 1). HeLa cells were used as a positive control (17). At different times thereafter (15, 30, 45, and 60 min), 0.8 ml of ice-cold medium was added and the cells were centrifuged (5,000 × *g* for 1 min at 4°C). The supernatants were subjected to titer determination on KB cell monolayers to determine the amounts of unbound virus (PFU per milliliter). The kinetics of virus-receptor interaction was analyzed by calculating the percentage of unattached virus at each time point.

Detection of sense and antisense viral RNA. Genomic sequences common to all CVBs were searched for by reverse transcription-PCR (RT-PCR). Total RNA was extracted from 10^6 infected cells by the guanidinium thiocyanate method, precipitated with isopropanol, washed in 70% ethanol, and dissolved in reagent-grade water treated with diethylpyrocarbonate. cDNA synthesis of CVB RNA sequences was done with picornavirus-specific primers selected from the conserved 5′ untranslated region of the genome (34). RT was carried out for 60 min at 42°C and followed by reverse transcriptase inactivation at 95°C for 2 h. cDNA of the genomic (sense)-strand RNA was obtained by priming the RT step with the downstream 3′ primer (5′-CATTCAGGGGCCGGAGGA-3′); cDNA of the replicative (antisense) strand RNA was produced by priming the RT step with the upstream 5′ primer (5′-AAGCACTTCTGTTTCC-3′). The reaction mixture (20 μl) contained 1 μg of RNA, buffer with nucleotides (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 5 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate [dNTP]), 20 U of RNase inhibitor, 50 U of Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Norwalk, Conn.), and 40 pmol of the appropriate primer (Genset, Paris, France). Then 2 μl of the RT product was subjected to 35 cycles of amplification in a thermal cycler (type 2400; Perkin-Elmer). The PCR mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 40 pmol each of the minus and plus primers, and 2 U of *Taq* polymerase (Perkin-Elmer). The times and temperatures used for denaturation, annealing, and extension were 30 s at 95°C, 30 s at 40°C, and 30 s at 72°C, respectively. The amplification products (305 bp) were analyzed in a 2% agarose gel with a 100-bp DNA molecular size ladder (Bio-Rad, Hercules, Calif.) for reference.

In situ hybridization. The proportion of infected MC containing CVB-3 and CVB-5 RNA was evaluated by in situ hybridization (32). Acutely or chronically infected MC were cultivated in chamber slides (Nunc, Naperville, Ill.). The cultures were washed twice with Hanks' balanced salt solution and fixed with Histochoice (Amresco, Solon, Ohio) for 20 min at 4°C. Before hybridization, the slides were treated for 5 min at 65°C with 70% formamide in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and for 60 min at room temperature with hybridization mixture (50% formamide, 1× Denhardt's solution, 4× SSC, 5% dextran sulfate, 0.5 mg of herring sperm DNA per ml, 0.25 mg of yeast tRNA per ml). Then 30 μl of this solution containing a biotinylated picornavirus-specific genomic probe (0.1 pmol/μl) (34) was added to each slide. The slides were incubated at 37°C for 18 h, washed twice for 30 min at room temperature in 2× and then twice in 1× SSC followed by 30 min at 37°C in 0.5× SSC and 30 min at room temperature in 0.1× SSC. They were then incubated for 30 min at 37°C in 4× SSC–5% skim milk and washed for 5 min in 4× SSC containing 0.25% Tween 20. The hybridized probe was revealed by staining with fluorescein-labeled avidin (Oncor, Gaithersburg, Md.) for 15 min at 37°C; the reaction was enhanced with anti-avidin antibody (Oncor). The slides were examined with an epifluorescence Olympus BX60 microscope (Olympus Optical Co., Tokyo, Japan).

Evaluation of cytokine synthesis and release in MC cultures. Cytokine release in conditioned medium from uninfected and infected MC was measured. Samples of supernatants were taken 3, 6, and 9 days after plating (reported data refer to day 6). As a positive control, MC cultures were exposed to lipopolysaccharide (LPS; 100 ng/ml) from *Escherichia coli* O11:B4. Cytokine levels were measured by immunoenzyme assays (interleukin-1β [IL-1β], tumor necrosis factor alpha [TNF-α], platelet-derived growth factor A/B [PDGF-A/B], and transforming growth factor β1/2 [TGF-β1/2] assays [R&D Systems, Minneapolis, Minn.]; IL-6 and IL-8 assays [Biosource, Camarillo, Calif.]; beta interferon [IFN-β] assay [Medgenix, Brussels, Belgium]). Cytokine-specific mRNAs were detected in total RNA extracted as described above. A 1-μg portion of RNA was treated with 6 U of RNase-free DNase for 1 h at 37°C and then for 5 min at 94°C. cDNA was obtained with random hexanucleotides (Clontech, Palo Alto, Calif.) or 3′-specific primers. RT was performed as described above. cDNA was subjected to 35 cycles of amplification with cytokine mRNA-specific primer pairs (Amplimer Set; Clontech). The PCR mixture (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 20 pmol each of 5′ and 3′ primers, and 2 U of *Taq* polymerase (Perkin-Elmer). The times and temperatures for denaturation, annealing, and extension were 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C, respectively. In the case of TGF-β1/2 mRNAs, PCR was performed for only 20 cycles to detect cytokine-specific transcripts under conditions of linear amplification (as determined in preliminary experiments). As a control reaction, RT-PCR of β-actin mRNA was carried out with all samples.

RESULTS

Comparative susceptibility of human renal cells to CVBs. Primary cultures of MC, GEC, and PTEC derived from five

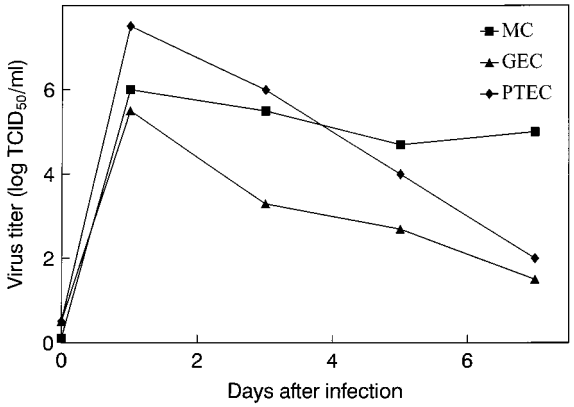

FIG. 1. Production of CVB-3 in primary cultures of human MC, GEC, and PTEC. Virus titers obtained in a single experiment are expressed as $TCID_{50}$ on KB cells. Consistent results were obtained with cells derived from five different donors.

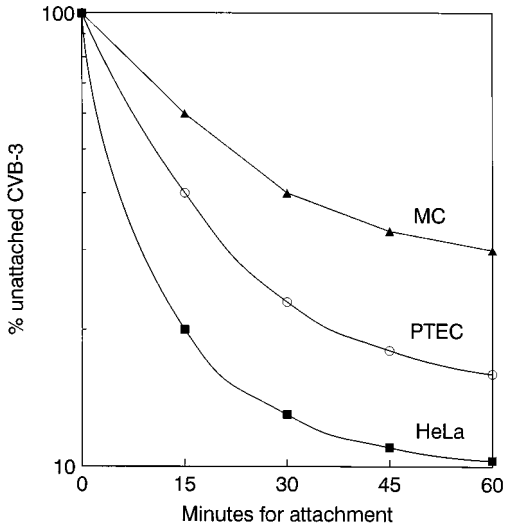

FIG. 2. Evaluation of the expression of CVB receptors in human renal cells. The kinetics of CVB-3 attachment to MC, PTEC, and HeLa cells were studied; HeLa cells are known to be positive for a common receptor specific to CVBs. The percentage of unattached virus was calculated by comparing at different times the PFU of unbound CVB-3 with the titer of virus present in the inoculum. Virus production was determined by a plaque assay on KB cells.

different donors were infected with CVBs, and virus production was monitored for 7 days. Primary cultures of the three different histotypes were susceptible to productive infection by the six CVBs. Virus titers peaked at 24 to 48 h p.i. and decreased thereafter. The presence of viral genomic sequences on day 1 p.i. (data not shown) confirmed the susceptibility of renal cells to CVBs. However, the kinetics of viral production and CPE induction were different in the three renal cell types. Figure 1 is representative of the kinetics of CVB-3 infection in MC, GEC, and PTEC. Cultures of PTEC were highly susceptible to all viral serotypes, showing cell rounding and detachment within 12 to 24 h p.i. Complete cytolysis occurred at 48 to 96 h p.i.; high virus titers were produced. Comparable results were obtained in GEC cultures. The viral titers were, however, slightly lower than those obtained in PTEC, and lysis of monolayers occurred in 5 to 7 days. In contrast, MC cultures produced virus titers 10- to 100-fold lower than those obtained in PTEC (Table 1) and CPE was observed in only 50 to 60% of cells. MC that survived CVB infection continued to proliferate and maintained virus production. Differences in CVB permissiveness were confirmed by the observation that whereas virus yields were not modified in PTEC by decreasing the MOI from 5 to 0.2, this slight reduction had significant effects in MC (Table 1).

The expression of CVB receptors was evaluated in renal cells. Comparison of the rate of CVB-3 binding to different cell types demonstrated that MC express lower levels of CVB-specific receptors than PTEC do. In fact, at all times analyzed, the percentage of unbound virus was higher in MC than in PTEC (e.g., 25 to 30% unbound virus in MC versus 13 to 18%

TABLE 1. Release of infectious viral progeny in primary cultures of renal MC and PTEC exposed to CVBs at different MOIs

Virus	Viral titer[a] at the following MOI in:					
	MC			PTEC		
	5	1	0.2	5	1	0.2
CVB-1	6.0	5.0	3.9	7.1	6.9	6.8
CVB-2	5.5	4.3	3.0	6.7	6.0	5.8
CVB-3	6.0	5.3	3.7	7.5	7.0	6.9
CVB-4	5.3	4.3	3.0	7.0	6.5	6.3
CVB-5	6.0	5.3	3.3	7.2	6.9	6.5
CVB-6	5.0	3.9	2.5	6.5	6.0	5.8

[a] Virus titers are expressed as $\log_{10}$ $TCID_{50}$ per milliliter on day 1 p.i.; the values represent mean titers in the supernatants of triplicate cultures (24-well microplates) obtained from three different donors. The standard deviation (not shown) was consistently lower than 0.5 $\log_{10}$ $TCID_{50}$/ml.

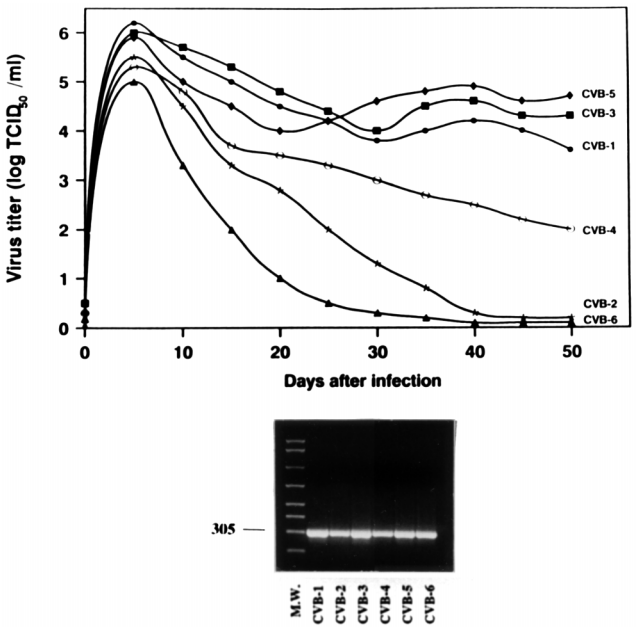

FIG. 3. Virus production in MC persistently infected with CVBs. (Top) The titer of each virus ($TCID_{50}$) in the supernatant was determined every 5 days of subculture. Values represent the mean of titers obtained in three independent experiments (standard deviations were lower than 0.7 $\log_{10}$ $TCID_{50}$ [not shown]). (Bottom) Agarose gel electrophoresis of amplicons specific to the negative strand of CVB genomes. Strand-specific RT-PCR was carried out on total RNA taken from MC infected for 15 days with CVB types 1 to 6. M.W., molecular size markers (100-bp DNA molecular size ladder). Equivalent expression of β-actin mRNA was detected in all samples.

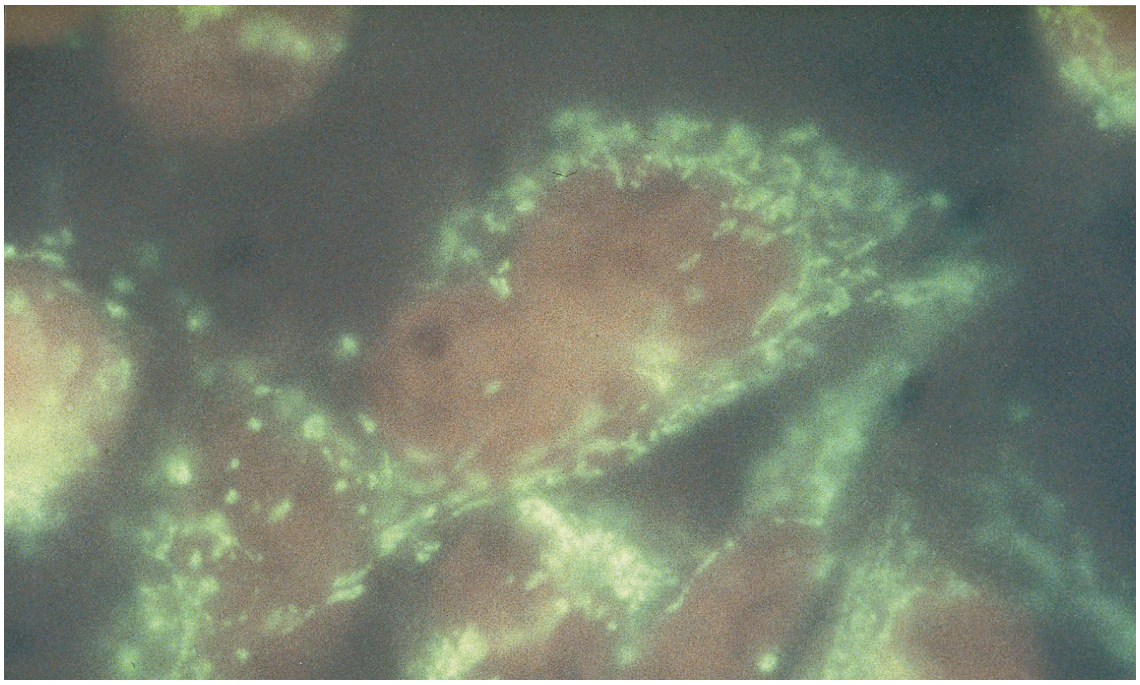

FIG. 4. In situ hybridization shows fluorescent cytoplasmic staining for sense-strand RNA of CVB-3 in human glomerular MC. Cell monolayers were fixed with Histochoice 5 days p.i. and hybridized with a biotinylated probe. The reaction was revealed with fluorescein-labeled avidin. Magnification, ×600.

in PTEC at 60 min). HeLa cells showed the highest binding capacity (Fig. 2).

Persistent infection of glomerular MC. In preliminary experiments, CVB infection of MC did not induce complete cytolysis and virus production was not shut off by two serial subcultures. Virus replication thus appeared to persist in MC beyond the acute phase of infection. This observation was confirmed by detecting negative-strand CVB RNA 15 days p.i. (Fig. 3, lower panel). As shown in Fig. 3 (upper panel), continuous replication of CVB-1, CVB-3, CVB-5, and CVB-4 (at lower levels) was detected in MC cultures for more than 50 days. Production of CVB-2 and CVB-6 lasted 35 and 30 days p.i., respectively. Persistent MC infection occurred in the absence of overt CPE. The trypan blue exclusion assay demonstrated that MC viability was only slightly influenced by virus replication since the percentage of viable cells ranged constantly between 80 and 90%. The relative quantity of positive and negative RNA strands, compared by serial end point dilution, indicated that the amount of negative strand was 50- to 100-fold smaller than the amount of positive strand (not shown). This suggests that CVB persistence in MC is not due to a defect in the control of viral genome synthesis, as reported in other cases (18). In situ hybridization confirmed the presence of CVB RNA in the cytoplasm of infected MC (Fig. 4). In the acute phase of infection with CVB-3 and CVB-5, viral replication occurred in 50 to 60% of the MC. In contrast, during persistent infection, only 5 to 10% of MC contained viral genomes. This suggests that CVB persistence is established by a carrier-state mechanism, as reported for lymphoid, myocardial, fibroblastoid, and endothelial cells (5, 16). Since the production of antiviral mediators can be involved in the initiation and maintenance of CVB persistence (16, 29), we investigated the expression of IFN-β and IFN-γ in MC. While we found no evidence of IFN-γ synthesis, we observed that both uninfected and infected MC express IFN-β-specific mRNA (Fig. 5). However, IFN-β was released at extremely low levels (i.e., 2 to 5 IU/ml). No evidence of IFN-β synthesis was found in PTEC and HeLa cells (data not shown).

Effects of persistent CVB infection on MC functions. MC play a prominent role in the pathogenesis of renal disease. Since activation of cytokine pathways in MC is associated with glomerulonephritis (51), we evaluated cytokine expression in CBV-infected MC. Table 2 compares the production of IL-1β, IL-6, IL-8, TNF-α, PDGF-A/B, TGF-β1, and TGF-β2 in uninfected MC and in MC persistently infected with CVB-3. As a control for the ability of infected cells to secrete cytokines, parallel experiments were carried out with cultures stimulated with LPS. Persistently infected cells retained the ability to respond to exogenous LPS, but no significant differences were seen in the release of IL-1β, IL-6, IL-8, and TNF-α. CVB-3 infection induced de novo the release of PDGF-A/B; the response was not further enhanced by LPS (Table 2). A moderate but significant enhancement of TGF-β1/2 was associated with CVB-3 persistence in MC. Comparable results were obtained in MC infected with CVB-5. Interestingly, in both un-

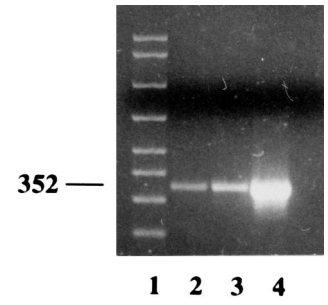

FIG. 5. RT-PCR analysis of IFN-β transcripts in renal MC. cDNA synthesis from total RNA was primed with a downstream primer specific to IFN-β mRNA. Lanes: 1, molecular size markers (100-bp DNA molecular size ladder); 2, uninfected MC; 3, CVB-3-infected MC; 4, positive control of IFN-β mRNA.

TABLE 2. Release of different cytokines in cultures of uninfected and CVB-3-infected renal MC

Cytokine	Cytokine level (pg/ml) in[a]:			
	Uninfected MC[b]		CVB-3-infected MC[c]	
	Unstimulated	+ LPS	Unstimulated	+ LPS
IL-1β	<5	112 ± 51	<5	155 ± 80
IL-6	830 ± 230	1,669 ± 430	860 ± 339	1,757 ± 391
IL-8	630 ± 255	1,536 ± 376	795 ± 320	1,660 ± 270
TNF-α	35 ± 15	44 ± 18	33 ± 19	39 ± 11
PDGF-A/B	<10	130 ± 53	550 ± 132[d]	584 ± 159[d]
TGF-β1	123 ± 67	663 ± 129	373 ± 103[d]	677 ± 202
TGF-β2	103 ± 38	503 ± 65	313 ± 95[d]	557 ± 177

[a] Cytokine levels in culture supernatants taken after 6 days of incubation were measured by imunoassay. The results are means ± standard deviations of three or four cultures.
[b] Uninfected cultures not stimulated or stimulated with LPS (100 ng/ml for 3 days).
[c] Cultures persistently infected with CVB-3 for 30 days. The cultures were not stimulated or were stimulated with LPS, as above.
[d] Significantly different from the relevant uninfected control group ($P < 0.01$).

infected and infected MC, LPS enhanced the expression of TNF-α transcripts (data not shown) but failed to induce the release of this cytokine.

Persistent replication of CVB-3 and CVB-5 in MC was found to induce the expression of PDGF-A/B mRNA (Fig. 6). The release of PDGF-A/B was induced early after acute infection with all CVBs (i.e., 1 day p.i.) and continued thereafter.

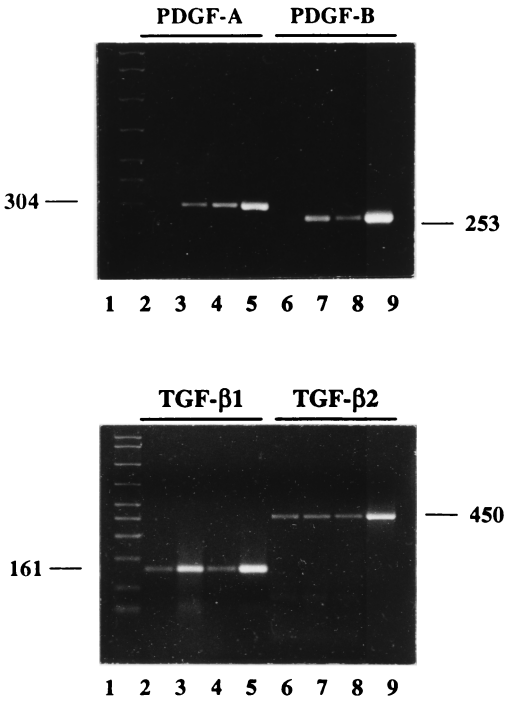

FIG. 6. Expression of mRNA transcripts for PDGF-A/B and TGF-β1/2 in uninfected and CVB-infected MC. Total cellular RNA was analyzed by RT-PCR 15 days p.i. Lanes: 2 and 6, uninfected cultures; 3 and 7, CVB-3-infected cultures; 4 and 8, CVB-5-infected cultures; 1, molecular size markers; 5, positive controls for PDGF-A (upper panel) and TGF-β1 (lower panel); 9, positive controls for PDGF-β (upper panel) and TGF-β2 (lower panel). The RT reaction was primed with cytokine-specific downstream primers. As described in Materials and Methods, amplicons of TGF-β1/2 were obtained with only 20 PCR cycles to compare the amounts of specific transcripts under conditions of linear amplification.

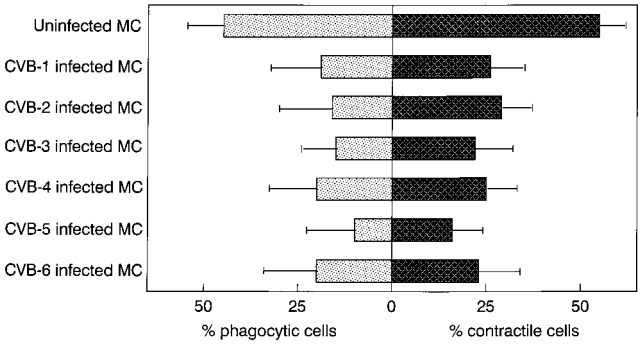

FIG. 7. Effect of CVB infection on the phagocytic and contractile properties of renal MC. At 15 days p.i., functional assays were carried out on CVB-infected and uninfected MC. The percentage of phagocytic cells was evaluated independently from the numbers of IgG-coated beads per cell. MC were considered contractile when they showed new or increased wrinkles 30 min after treatment with angiotensin II. Experiments were carried out in triplicate; results were evaluated by two independent observers.

This cytokine is mitogenic for MC and plays a pathogenic role in glomerulonephritis (51, 60).

CVB infection enhanced the release of TGF-β1/2, a regulator of extracellular mesangial matrix deposition (27). Under conditions of linear amplification (i.e., 20 amplification cycles), it was found that CVB-3 infection upregulated TGF-β1 transcripts (Fig. 6). After infection with CVBs, TGF-β1 and TGF-β2 levels started to rise from day 3 onward. However, in the case of CVB-3, the enhanced release of TGF-β2 was not associated with the increased expression of specific mRNA transcripts (Fig. 6). Similarly, in the case of CVB-5 infection, the enhanced release of TGF-β1 was not associated with an increased expression of specific mRNA transcripts. Thus, it appears that CVB infection may favor the release of preformed TGF-β.

MC possess both contractile and phagocytic properties that play important roles in glomerular hemodynamics and in handling macromolecules and immune complexes (24, 46). Phagocytosis assays with IgG-coated latex beads demonstrated that 40 to 50% of uninfected MC were able to ingest these particles. Only 10 to 20% of MC maintained phagocytic functions on day 15 after infection with the six CVBs (Fig. 7), and the number of ingested particles per cell was significantly reduced (15 ± 4 and 3 ± 2 in uninfected and infected MC, respectively). MC are characterized by a contractile response to a number of vasoactive agents. In our experiments, exposure of MC to angiotensin II on a silicone rubber surface produced new and increased wrinkles in more than 50% of uninfected cells whereas wrinkling was significantly reduced in CVB-infected MC cultures (Fig. 7).

DISCUSSION

A correlation between acute viral infections and renal damage has been established in systemic infections caused by influenza virus, mumps virus, CMV, varicella-zoster virus, and Epstein-Barr virus (35, 54, 62, 72). Viruria accompanying viremia was a common feature. Viruria is considered secondary to virus shedding from kidney cells rather than being due to glomerular filtration, since viruses are much larger than filterable macromolecules. This implies that virus shedding into urine is a result of virus replication in kidney cells. Renal injury associated with acute viral infection is usually associated with a transient reduction of renal function (70). In rare cases, such as

the hemorrhagic fever due to Hantaan virus, a bunyavirus, tubular necrosis and proliferative/exudative glomerulonephritis occur and frequently evolve to chronic renal failure (41). It has been reported that chronic infections due to polyomaviruses, CMV, hepatitis B virus, and HIV are associated with renal damage in animals and in humans (1, 36, 44, 62, 66). Proliferative and nonproliferative glomerulonephritis has been ascribed to the immune response against viral antigens or, alternatively, to a direct effect of the viruses on resident glomerular cells (31, 70). The above pathogenic mechanisms are, however, not mutually exclusive. For instance, glomerular injury induced by CMV (a major renal pathogen in congenitally infected infants and in renal allograft recipients) is related both to immune complexes containing CMV antigens and to CMV-directed macrophage influx into renal tissue (62). An additional pathogenic factor is represented by cytokines. In fact, cytokines released from infected mesangial or tubular cells can direct macrophage migration into renal glomeruli and interstitial tissue (65).

CVBs often cause subclinical infections characterized by acute viremia that follows their initial replication at the portal of entry (56). The variable clinicopathological expression of CVB-induced disease appears to depend mainly on virus tropism (17, 33) but also on the acute or persistent nature of infection. Enteroviruses, in fact, can cause persistent infections of the heart (38, 48), skeletal muscle (18), and central nervous system (63). Persistent CVB replication has also been observed in different types of cultured cells, i.e., myocardial fibroblasts (29), lymphoid cells (43), and vascular endothelial cells (16). In some of the above cell types, CVBs appeared to alter cell functions and to stimulate the production of cytokines (16, 30, 67).

Sporadic reports indicate that CVBs can cause glomerulonephritis in humans (11, 25); these agents also appear to cause nephritis in mice (40, 49, 50). Using in vitro models, we investigated the susceptibility of renal cells to CVBs. Pure cultures of glomerular MC, GEC, and PTEC were studied.

MC are specialized pericytes of vascular smooth muscle origin that have several functions in glomerular physiology: (i) control of glomerular hemodynamics through contractile activity (13); (ii) clearance by phagocytosis of macromolecules and immune complexes passing from the capillary lumen into the mesangium (3, 24, 46, 64); (iii) production of extracellular matrix (27); and (iv) release of autacoids and proinflammatory cytokines in response to inflammatory stimuli (19, 46, 59). Mesangial deposits of Igs (a frequent finding in glomerulonephritis) may reflect defective clearance due either to excessive deposition or to defective phagocytosis (24, 64). A variety of glomerular disorders are characterized by expansion of the mesangial matrix and by an increased number of glomerular cells, partly as a result of MC proliferation (51).

GEC (i.e., podocytes) are often involved in glomerular injury (37). Retraction of the foot processes of podocytes from capillary loops is a basic reaction pattern in glomerular damage. It is also known that these cells have a negatively charged glycocalyx and express β1-integrins that bind them to the glomerular basement membrane (20, 39). Virus infections frequently alter cytoskeleton functions and downregulate the expression of surface molecules. In vivo, GEC alterations are frequently associated with proteinuria and contribute to the progression of glomerular damage (37).

The proximal tubule is responsible for the reabsorption of about 60% of the glomerular ultrafiltrate (42). PTEC are metabolically very active and possess a tall brush border, numerous mitochondria, and a well-developed endocytic-lysosomal apparatus that plays a role in the reabsorption of low-molecular-weight proteins filtered by the glomerulus (71). We have shown that cultured PTEC fix complement through the alternative pathway (7).

Little is known about the turnover of resident glomerular and tubular cells. Under physiological conditions, these cells have a low rate of replacement, but after renal injury they acquire the ability to proliferate (10). MC and GEC proliferation has been shown in the case of immune system-mediated kidney damage, and these cells contribute, by producing extracellular matrix, to the development of renal sclerosis (36, 50). Tubular epithelial cells represent the main target of the pathologic events inducing acute tubular necrosis, such as toxic, ischemic, or viral injuries (10, 52). However, these cells have the ability to proliferate and restore the normal morphological and functional conditions of tubules (10).

Our results demonstrate that human renal cells of three different histotypes are permissive to the six CVBs and that epithelial cells of glomerular and tubular origin are more prone than MC to CVB-induced CPE. While GEC and, in particular, PTEC demonstrated massive CPE within 24 to 72 h p.i., MC supported continuous CVB production in the absence of overt CPE. In MC, infection stimulated the release of PDGF-A/B and TGF-β1/2 and reduced the phagocytic and contractile activity of these cells.

Viral replication in PTEC—which are particularly sensitive to CVB-induced CPE—might be responsible for the sustained viruria and transient renal dysfunction that have been reported occasionally in acute viral infection (11, 25, 35, 54). In fact, the renal tubule represents the main site of injury in most cases of acute renal failure (52). GEC also were highly susceptible to CVBs. In contrast, MC produced CVB titers lower than those observed in epithelial cells and were capable of supporting the chronic replication of these viruses in the absence of overt CPE.

In principle, the distinct susceptibility of human renal cells to CVBs may be explained by the differential expression of CVB-specific receptors. We found that MC had a lower ability than PTEC to bind CVBs. Since factors other than receptor expression also play a role in susceptibility to picornaviruses (53), it is likely that the reduced sensitivity of MC is associated with the constitutive synthesis of IFN-β observed in these cells. Endogenous synthesis of IFN-β has already been shown to contribute to CVB persistence in targets such as human endothelial cells and myocardial fibroblasts (16, 29). Infectivity assays and detection of antisense-strand RNA showed that CVBs were continuously produced in persistently infected MC cultures.

Persistent infection of MC failed to alter the production of IL-1, TNF-α, IL-6, and IL-8. Cultured MC are known to produce TNF after stimulation with either IL-1, the terminal complement components (58), or platelet-activating factor (8). In contrast, TNF production is not induced by LPS, since the concomitant production of prostaglandin E_2 limits TNF synthesis (4, 58). In our experiments, uninfected and CVB-infected MC showed an enhanced expression of TNF transcripts after LPS stimulation. However, gene expression was not accompanied by a significant increment of TNF-α release, suggesting that translation or secretion of TNF is defective in MC.

Infection of MC appeared to induce de novo the expression of PDGF-A/B transcripts. PDGF is known to stimulate MC proliferation in an autocrine manner (59), and histopathological studies showed that PDGF transcripts and PDGF receptors are expressed in proliferative glomerulonephritis (60). Interestingly, CVB-4 has also been shown to cause proliferative glomerulonephritis in mice (50).

TGF-β plays a defined pathogenic role in glomerulosclero-

sis. For instance, transfection of renal cells with cDNA coding for TGF-β leads to glomerulosclerosis and renal failure in mice (9). Interestingly, CVBs stimulated the release of TGF-β1/2 in MC. This phenomenon was only in part associated with the enhanced expression of specific transcripts, suggesting that infection somehow triggers the secretion of stored cytokine. As in other organs, TGF-β stimulates the synthesis of extracellular matrix and inhibits the degradation of matrix proteins in the kidneys (9). Persistent infection also reduced the contractile and phagocytic activity of MC. This finding is consistent with the evidence of reduced uptake and transport of colloidal carbon in the mesangium of mice developing CVB-induced glomerulonephritis (40).

In conclusion, human renal cells of glomerular and tubular origin are highly permissive in vitro to the six CVBs. In contrast, MC, but not the other cells derived from the kidney cortex, can support both acute and continuous replication of CVBs. In the latter cells, infection appears to stimulate the production of factors that play a pathogenic role in progressive nephropathy. Clinical studies are needed to evaluate whether CVB infections are involved in the pathogenesis of idiopathic forms of glomerulonephritis.

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