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MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells

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MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithelial cells. Proximal tubular (PT) epithelial cells express MHC class II (Ia) antigens in immunologically-mediated renal injury. To study the role of PT as accessory cells, we generated several murine PT-like epithelial cell lines by transformation with origin-defective SV40 DNA. These transformed cell lines display typical alkaline phosphatase and γ-glutamyl-transpeptidase enzyme activity, proliferation to epidermal growth factor (EGF) and sodium-dependent glucose uptake. Clonal lines of transformed tubular cells from both normal C3H/FeJ and autoimmune MRL-*lpr* mice do not constitutively express Ia antigens or mRNA for class II. However, stimulation with recombinant interferon-γ (rIFN-γ) induces Ia mRNA and surface product in the cell lines. These Ia-positive cells can process and present hen egg-white lysozyme (HEL) to antigen-specific Ia^k-restricted T cell hybrids. Unstimulated tubular cells do not express detectable IL-1α, IL-1β, TNF-α, or IL-6 mRNA. However, stimulation with IL-1α or LPS induces TNF-α transcripts. We conclude that these cell lines have characteristics most consistent with a proximal tubular origin. They also bear characteristics of accessory cells such as processing and presentation of antigen and TNF-α gene expression. We speculate that PT have the capacity to participate in the pathogenesis of immune renal injury.

Proximal tubular (PT)[1] epithelial cells in the kidney express major histocompatibility (MHC) class II (Ia) antigens in various inflammatory conditions, such as allograft rejection [1], graft-versus-host disease [2], and lupus nephritis [3]. We have previously shown that the PT of autoimmune MRL-*lpr* mice display focal Ia expression by two months of age, preceding glomerulonephritis [4]. Early focal PT Ia expression could be an important mechanism in the initiation of autoimmune renal injury.

In cells of the immune system, Ia antigens play a fundamental role in the presentation of foreign or autoantigens to helper T cells. Some cells of non-hematopoietic lineage can also express Ia antigens and function as accessory cells. Chondrocytes [5] and enterocytes [6] for example can process and present antigen to T cells. Other non-hematopoietic cells such as keratinocytes [7] and pancreatic β-cells [8] induce antigen-specific unresponsiveness in T cells. Thus, many non-immunologic cells which express Ia antigens can interact with cells of the immune system.

To define the role of renal tubular epithelial MHC class II expression in renal injury, we generated PT-like epithelial cell lines from renal cortical tubular cells by transformation with origin-defective SV40 DNA. We show that clonal lines of transformed epithelial cells retain their role as solute transporting epithelium and have differentiated characteristics of accessory cells, such as processing and presentation of antigen. Moreover they have the capacity to express TNF-α transcripts. We extrapolate that these "immune" functions may allow the PT to participate in the pathogenesis of immune renal injury in vivo.

Methods

Materials

Tissue culture media and reagents were from Gibco (Grand Island, New York, USA), and chemicals from Sigma (St. Louis, Missouri, USA). Monoclonal antibodies (MAb) for $I\text{-}A^k$ (clones 10-2.16 and 14-4-4S, American Type Culture Collection [ATCC], Rockville, Maryland, USA) and $I\text{-}A^d$ (clone MK-D6) were obtained by affinity purification of supernatants over protein A-Sepharose CL-4B columns (Pharmacia, Piscataway, New Jersey, USA). The rat anti-murine IFN-γ MAb R4-6A2 (ATCC) was purified over recombinant protein G-Sepharose 4B (Zymed, South San Francisco, California, USA). MAbs for cytokeratin (PKK1) and vimentin were purchased from Labsystems (Helsinki, Finland). A MAb for the large T antigen of SV40 (419) was provided by Dr. David Livingston (Harvard Medical School). Supernatants from J558L cells transfected with the murine IL-4 gene (Dr. Robert Tepper, Harvard Medical School) were used as source for rIL-4. The origin-defective SV40 mutant 6-1 has been described [9, 10]. This mutated SV40 DNA lacks six base pairs at the SV40 origin of DNA replication, preventing viral self-replication. The source and nature of the genomic probe for $A_\alpha{}^b$ [11] and the cDNAs for IL-1α [12], IL-1β [13] and TNF-α [14] have been described elsewhere [4,

[1] Abbreviations used in this paper: PT, proximal tubules; SV40, simian virus 40; MHC, major histocompatibility complex; MRL/MpJ-*lpr*/*lpr*, MRL-*lpr*; HEL, hen egg-white lysozyme; APC, antigen presenting cell.

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15, 16]. A murine IL-6 cDNA clone sharing extensive sequence homology with the published sequence [17] was obtained from Genetics Institute (Cambridge, Massachusetts, USA). An 800 bp *Pst*I fragment was used for hybridization.

Generation of SV40 transformed tubular epithelial cell lines

Single cortical tubular cell suspensions of freshly dissected kidney cortices from normal two-month-old C3H/FeJ (H-2^k) and from two- or five-month-old autoimmune MRL-*lpr* (H-2^k) were prepared by collagenase dispersion (cls II, 10 mg/ml; Worthington, Freehold, New Jersey, USA), sequential sieving through steel meshes (250 μm, 150 μm, 75 μm, and 38 μm), and washing in Hanks' balanced salt solution (HBSS). Cells were grown to subconfluence on collagen-coated 50 mm plates for six days in modified K1 media (1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and Ham's F12, containing the following additives: 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 5% fetal calf serum [FCS], 5 μg/ml insulin, 5 μg/ml transferrin, 1 ng/ml prostaglandin E_1, 5×10^{-11} M triiodothyronine, 10^{-8} M sodium selenite, 5×10^{-8} M hydrocortisone, 25 ng/ml epidermal growth factor; EGF). Cells in primary culture displayed strong alkaline phosphatase reactivity, a brush border marker enzyme of PT. Using the calcium-phosphate co-precipitation method [18], the cells were transfected with origin-defective SV40 DNA mutant 6-1 in pMK16 (20 μg per 50 mm culture dish) and maintained with weekly changes of fresh modified K1 media. Distinct foci of outgrowing cells became apparent after six weeks. These foci of clonal cells were transferred to 24-well plates and were then passaged by trypsinization (trypsin 0.5 μg/ml and EDTA 0.53 mM). PT cell lines from C3H/FeJ mice were designated C1, and those from MRL-*lpr* mice, M1 and M3. Cells were subcloned in 96-well plates by the limiting dilution technique to exclude the possibility of a mixed cell population. Briefly, using an irradiated (3000 rads) feeder layer of the same cell type, cells were plated out in 96-well plates at 0.3, 1 and 10 cells/well. Positive wells from the lowest dilution with less than 10% positive wells were selected. The subclones retained alkaline phosphatase activity and were designated C1.1, M1.1 and M3.1.

Enzyme cytochemistry

Confluent monolayers of SV40 transformed cells were assessed for brush border enzyme activity. Alkaline phosphatase activity was demonstrated by incubating unfixed cells for 60 minutes with an alkaline solution of naphthol AS-MX phosphate containing Fast Blue RR salt (Sigma, kit # 85). γ-glutamyl-transpeptidase activity was visualized according to Rutenburg et al [19] by incubating the unfixed cells for 60 minutes in a solution containing 0.125 mg/ml γ-glutamyl-4-methoxy-β-naphthylamide, 0.5 mg/ml glycylglycine, and 0.5 mg/ml Fast Blue BBN, followed by postcoupling with 100 mM copper sulfate. Reactions were scored positive or negative.

Immunofluorescence

Transformed cells were grown on glass coverslips to confluence and fixed for two minutes with 2% paraformaldehyde. The cells were then washed twice with 50 mM Tris buffer (pH 7.6), incubated with diluted (1:10) MAb for cytokeratin, vimentin or large T antigen, washed twice with 50 mM Tris buffer, and then incubated with fluorescein-conjugated affinity-purified goat anti-mouse IgG (F(ab')$_2$ fragment (Cappel, Cochranville, Pennsylvania, USA). After two washes with Tris buffer, the coverslips were mounted with glycerol-gelatin and examined by fluorescence microscopy.

Proliferation assays

Growth characteristics of SV40 transformed cells in response to EGF were studied by [^{3}H]thymidine incorporation and also using a colorimetric assay [20]. For both assays, cells (5×10^3 or 10^4 cells/well) were placed in collagen-coated 96-well plates and grown in DMEM/Ham's F12 (1:1) with or without EGF (25 ng/ml), containing 25 mM Hepes, penicillin (100 U/ml) and streptomycin (100 μg/ml). After 24 hours the cells were then pulsed with [^{3}H]thymidine (1 μCi/well) overnight and harvested using a PHD cell harvester (Cambridge Technology, Cambridge, Massachusetts, USA). For the colorimetric assay, which directly reflects the number of metabolically active cells in the wells, cells were grown in 96-well plates for 48 hours, followed by a four hour pulse with 50 μg of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Living cells cleave the tetrazoliuim ring from MTT, producing a blue formazan salt. An equal volume of 10% sodium dodecyl sulfate (SDS) was added for 12 hours to dissolve the insoluble formazan. The plates were read at 570 nm on a Dynatech microELISA reader, subtracting the OD at 650 nm as background.

PGE_2 synthesis

Transformed cells were grown to confluence for two to four days. The media was changed for 24 hours to media without additives. One hour before stimulation the media was changed once. The cells were then stimulated with angiotensin II (Ang II), arginine-vasopressin (AVP) or calcium-ionophore A23187 for 30 minutes. The supernatant was immediately assayed for PGE_2 using a direct competitive radioimmunoassay as previously described [21].

Cyclic AMP generation

SV40 transformed cells were grown as described above for PGE_2 synthesis. The cells were stimulated for 30 minutes with parathyroid hormone (PTH), AVP or forskolin. The supernatants were collected and assayed for extracellular cAMP using a commercially available kit (Amersham, Arlington Heights, Illinois, USA).

Sodium-glucose cotransport

Sodium-dependent glucose uptake was studied according to Stanton et al [22], using methyl α-D-glucopyranoside [^{14}C] (New England Nuclear, Boston, Massachusetts, USA), a non-metabolizable analogue of D-glucose. Transformed cells were grown in 6-well plates for two or three days until confluent. The cells were then washed three times with a sodium-containing solution (135 mM Na^+, 4 mM K^+, 1 mM Mg^{2+}, 1.8 mM Ca^{2+}, 5 mM Hepes, 5 mM L-alanine, 1.5 mM PO_4^{3-}, 0.5 mM methyl α-D-gluco-pyranoside), or a solution where the sodium was replaced by N-methyl-D-glucamine, or a sodium-containing solution containing 0.5 mM phloridzin. The cells were then incubated at 37°C for 15 or 60 minutes with 1.5 ml of the corresponding solutions containing 0.6 μCi methyl α-D-glucopyranoside [^{14}C]. The uptake was terminated by rinsing the wells three times with ice-cold stop solution (156 mM $MgSO_4$, 1

mM Tris, 1.6 mM Hepes, pH 7.6). The cells were permeabilized with 0.1 N NaOH for one hour, and the solution neutralized with 1 N HCl. The uptake in the solutions was counted by liquid scintillation spectroscopy. The total protein content in the solutions was determined by the Coomassie Blue dye binding method (Biorad, Richmond, California, USA).

RNA preparation

Total RNA was isolated from confluent monolayers of transformed cells using a single-step acid guanidinium isothiocyanate-phenol-chloroform extraction method [23]. The cells were lysed with a denaturing solution of 4 M guanidinium isothiocyanate (BRL, Gaithersburg, Maryland), 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol, and the lysate was directly extracted with phenol and chloroform. After isopropanol precipitation and 75% ethanol washes, the RNA was quantitated by measuring the OD at 260 nm.

Northern analysis

Total RNA (25 μg) was denatured with 1 M glyoxal and 50% dimethylsulfoxide, electrophoresed through a 1.5% agarose gel, and transferred onto Gene Screen nylon membranes (New England Nuclear, Boston, Massachusetts, USA). The RNA was baked onto the membrane at 80°C for four hours, and blots were prehybridized with 25 μg/ml denatured salmon sperm DNA and 25 μg/ml yeast tRNA in 50% formamide, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin, 0.04% Ficoll, 5x standard sodium citrate (SSC) and 1% SDS. Probes were radiolabeled by primer extension using random hexanucleotides [24]. Blots were hybridized for 16 to 24 hours at 42°C and washed under stringent conditions (twice at room temperature with 2xSSC, 1% SDS; twice at 65°C with 2xSSC, 1% SDS; and twice at 65°C with 0.1xSSC, 1% SDS). Following hybridization the blots were exposed to Kodak X-AR film. All blots were also rehybridized with β-actin to ensure that equal amounts of RNA were loaded for electrophoresis.

Flow cytometry

SV40 transformed cells were trypsinized, washed with HBSS, and stained for MHC class II antigens using MAbs 10-2.16 (anti I-A^k), 14-4-4S (anti I-E^k), or irrelevant MAb MK-D6 (I-A^d) as primary antibody. Fluorescein-conjugated affinity-purified goat anti-mouse IgG F(ab')$_2$ fragment was used as secondary step. Cells were analyzed with a Becton-Dickinson flow cytometer.

Antigen presentation

Transformed tubular epithelial cells were cultured for three days in the presence or absence of rIFN-γ (100 U/ml) for Ia induction. Antigen-presentation was then determined by using two previously-described T cell hybrids [25], which are Ia^k-restricted and specific for hen egg-white lysozyme (T hybrid C10, and a subclone of A2.2B2, the A2A2 hybrid). Such hybrids secrete interleukin-2 (IL-2) when co-cultured with Ia-matched antigen presenting cells (APC) in the presence of the appropriate foreign antigen. Varying numbers (3×10^3 to 10^5) of Ia-positive PT or control B cells (TA3 and 2B1 B cell hybridomas) were co-cultured with 10^5 T hybrid cells in 96-well plates with 50 μg/well of hen egg lysozyme (HEL, Sigma). After 24 hours, supernatants were collected and assayed for IL-2 content in a secondary culture using HT-2 cells, an IL-2 dependent T cell line. HT-2 cells (5×10^3) were cultured for 24 hours in the presence of serial dilutions of the primary culture supernatant. The degree of stimulation was determined visually (in arbitrary U/ml of IL-2) according to Kappeler et al [26] or by the incorporation of [^{3}H]thymidine into DNA six hours later. To study antigen processing, cells were pulsed with HEL and fixed with 1% paraformaldehyde (10 min at room temperature), or fixed and then exposed to HEL before culturing with the T cell hybrids.

Cytokine production

Transformed tubular epithelial cells were grown to confluence and stimulated for 24 hours with rIL-1α, rTNF-α, lipopolysaccharide (LPS; *S. minnesota*, Calbiochem, La Jolla, California, USA), rIL-4 or rIFN-γ. RNA was extracted, electrophoresed and blotted as described. Blots were hybridized as described above with specific cDNA probes for the generation of IL-1α, IL-1β, TNF-α, or IL-6 mRNA.

Results

Morphologic characterization of the transformed cell lines

Transformation of primary cortical tubular cell cultures with origin-defective SV40 DNA resulted in successful generation of uniform clonal cell lines with characteristics of PT (Fig. 1 and Table 1). Transformed tubular epithelial cell lines from C3H/FeJ (C1) and MRL-*lpr* (M1, M3) all form rapidly growing adherent monolayers. In addition, dome formation is detected, indicating a functional transporting epithelium. Positive nuclear staining for the SV40 large T antigen is detected in all cells, indicating transformation by SV40 DNA, and excluding the presence of untransformed cells. All cell lines display histochemical reactivity for alkaline phosphatase and γ-glutamyltranspeptidase, two brush border marker enzymes, and stain by immunofluorescence uniformly with intracytoplasmic cytokeratin, an epithelial cell marker, but not with vimentin, a mesenchymal marker. No macrophages are detected.

Growth characteristics

Cell growth was studied to determine whether growth factors such as EGF retain their ability to regulate proliferation. By phase microscopy the two cell lines examined (C1.1 and M3.1) proliferate more rapidly in the presence of EGF (25 ng/ml) than with control media (DMEM/F12 1:1) alone. As shown in Figure 2 (upper panel), both cell lines demonstrate enhanced [^{3}H] thymidine incorporation (1- to 3-fold in various experiments) in response to EGF, indicating DNA synthesis. M3.1 cells proliferate more slowly than C1.1 cells. These results were confirmed in a second assay for cell proliferation, MTT cleavage as determined by colorimetry (Fig. 2, lower panel). This assay directly reflects the number of metabolically active cells in the wells. Addition of either EGF, hormone mixture (insulin, transferrin, sodium selenite, hydrocortisone, PGE_1 and triiodothyronine) or 5% FCS to the control media results in enhanced cell growth. Combination of all three additives (EGF, hormone mixture and FCS; modified K1) results in the highest proliferation rate.

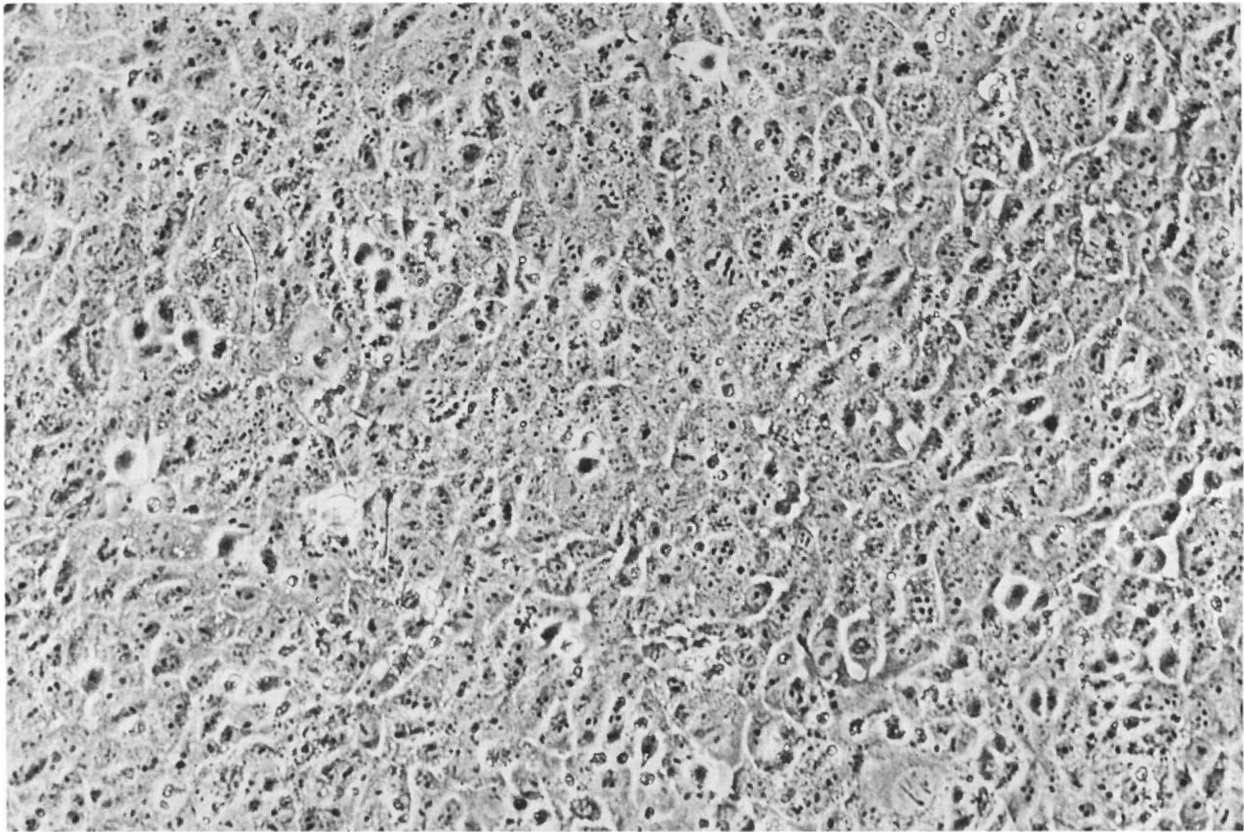

Fig. 1. *Phase micrograph of SV40 transformed tubular epithelial cells derived from MRL-*lpr *(M3.1), demonstrating homogeneous confluent monolayers (×200).*

Table 1. Morphologic characterization of SV40 transformed tubular epithelial cell lines

Cell type	*C1.1*	*M1.1*	*M3.1*
Histochemistry			
alkaline phosphatase	+	+	+
γ-glutamyl-transpeptidase	+	+	+
Immunocytochemistry			
SV40 large T antigen	+	+	+
cytokeratin	+	+	+
vimentin	−	−	−

Transformed cells were grown to confluence in 50 mm culture dishes for enzyme cytochemistry, and on glass coverslips for immunofluorescence. Key: −, negative; +, positive.

Functional characterization

PGE_2 synthesis and cAMP production. Transformed cells (C1) were studied for hormonal responsiveness (Table 2). Ang II (10^{-8} to 10^{-6} M) induces a dose-dependent increase in PGE_2 synthesis, whereas AVP (10^{-6}M) does not stimulate PGE_2 synthesis. Ang II-stimulated PGE_2 synthesis is blocked with indomethacin. Calcium-ionophore A23187 also induces PGE_2 synthesis. PTH weakly stimulates cAMP production. A stronger cAMP response is seen to AVP, suggesting that these tubular cells, unlike untransformed PT, have vasopressin receptors. Subcloned C1.1 also produce cAMP in response to both PTH and AVP (not shown), suggesting that the AVP response is not due to a contaminated distal cell type. Forskolin, a direct stimulator of adenylate cyclase also enhances cAMP synthesis in C1.1 (45 min stimulation: control, 1.1 ± 0.2 pmol/well; forskolin 10^{-4} M, 48.0 ± 0.0 pmol/well, $P < 0.001$).

Sodium-dependent D-hexose uptake. C1.1 and M3.1 cells were examined for sodium-glucose cotransport, a specific function of PT. Figure 3A shows one representative experiment of four in C1.1 cells. Both cell lines show uptake of the non-metabolizable analogue of D-glucose, methyl α-D-glucopyranoside [^{14}C]. Uptake is apparent at 15 minutes, and further increased at 60 minutes. Uptakes were lower than what has been reported in the literature for untransformed cells [22, 32]. The transport is inhibited by 0.5 mM phloridzine, a specific inhibitor of the sodium-glucose co-transporter. Uptake is also inhibited in sodium-free solution where the NaCl is replaced by N-methyl-D-glucamine. Figure 3B summarizes the inhibitory effect of phloridzine and sodium removal in C1.1 and M3.1 cells in three separate experiments.

Regulation of MHC class II expression

Unstimulated cells (C1, M1 and M3) do not display surface Ia or mRNA for class II, but can be induced to express Ia when stimulated with rIFN-γ (10 to 1000 U/ml for 3 days). Figure 4 (left panel) shows flow cytometric analyses of rIFN-γ stimulated C1, M1 and M3 cells. Both I-A (MAb 10-2.16) and I-E (MAb 14-4-4S, not shown) expression is induced with rIFN-γ in all three cell lines. No class II-specific mRNA is detected in

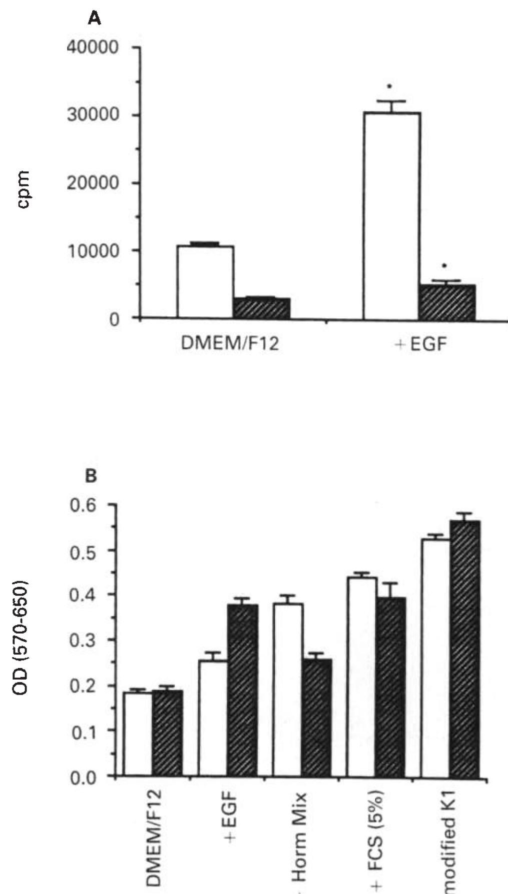

Fig. 2. *Proliferation assay of transformed tubular cells (C1.1 and M3.1).* Cells were placed in 96-well plates (□, C1.1, 5 × 10^3/well; ▨, M3.1, 10^4/well). **A.** [^{3}H]thymidine incorporation over 18 hr in C1.1 and M3.1 cells, grown for 24 hr in DMEM/F12 media (1:1) with or without EGF (25 ng/ml). **B.** MTT cleavage over 4 hr by C1.1 and M3.1 cells, grown for 48 hr in DMEM/F12 with either EGF (25 ng/ml), hormone mixture (insulin, triiodothyronine, PGE_1, hydrocortisone, sodium selenite and transferrin), FCS (5%), or in modified K1 media. Results are mean ± SE from quadruplicate determinations. * $P < 0.05$ (t-test).

unstimulated cells by Northern analysis (Fig. 4, right panel), but Ia transcripts can be induced with rIFN-γ.

Recombinant IFN-γ stimulated class II expression was further studied in C1 cells. The IFN-γ induced Ia expression is blocked with anti-murine IFN-γ MAb R4-6A2 (Fig. 5, lanes 1 to 4). Cycloheximide (0.5 to 5 μg/ml) also blocks IFN-γ stimulated Ia induction (Fig. 5, lanes 5 to 8), suggesting that protein synthesis is necessary for this induction. In addition, rIFN-γ stimulated Ia expression can also be inhibited by 10^{-4} M dexamethasone and hydrocortisone (Fig. 5, lanes 9 and 10). Hydrocortisone at a lower dose (5 × 10^{-5} M) inhibited the response partially (not shown).

The cell lines were stimulated with the other known Ia-inducing cytokines TNF-α and IL-4 (Fig. 6). Recombinant TNF-α (100 ng/ml) and rIL-4 (1000 U/ml) do not induce detectable Ia mRNA levels in C1 cells. Combining rTNF-α (100 ng/ml)

Table 2. PGE_2 and cAMP synthesis by SV40 transformed tubular cells

Sample	PGE_2 *pg/well*
Experiment 1	
control	6.5 ± 6.5
Ang II 10^{-8} M	0.0 ± 0.0
Ang II 10^{-7} M	144.5 ± 37.4[a]
Ang II 10^{-6} M	289.5 ± 19.7[a]
AVP 10^{-6} M	0.0 ± 0.0
Ang II 10^{-6} M + indomethacin (10^{-4} M)	0.0 ± 0.0
Experiment 2	
control	41.5 ± 3.2
A23187 10^{-5} M	192.5 ± 14.6[a]
Sample	**cAMP *fmol/well***
Experiment 3	
control	243 ± 11
PTH 10^{-10} M	304 ± 24
PTH 10^{-9} M	376 ± 26[a]
AVP 10^{-8} M	> 1024[a]

Transformed tubular cells (C1) were grown for 2 to 3 days in 24-well plates, washed once, and stimulated for 30 min. PGE_2 and extracellular cAMP (N = 4) were assayed in duplicates by radioimmunoassay as described in **Methods**. Results are mean ± SE.
[a] $P < 0.05$ when compared with respective controls (t-test).

and rIFN-γ (10 and 50 U/ml) does not potentiate Ia mRNA induction. LPS (1 to 5 μg/ml) does not affect Ia transcription.

Antigen presentation studies

Since one function of Ia molecules is to participate in antigen presentation to T cells of the $CD4^+$ phenotype, we sought to determine whether tubular epithelial cells have antigen presenting capacity. C1, M1 and M3 cells were examined for their ability to present antigen to HEL-specific, Ia^k-restricted T cell hybrids (Table 3). Class II negative tubular cells do not activate Ia^k-restricted C10 cells in the presence of HEL, whereas rIFN-γ induced Ia-positive tubular cells do activate C10 cells. In the absence of HEL, C10 cells are not activated by Ia positive APC. In three other experiments, IFN-γ induced C1 and M3.1 cells (3 × 10^3 to 10^5) were assayed for antigen presentation to another Ia^k-restricted, HEL-specific T cell hybrid (A2A2). Again, this hybrid releases IL-2 when co-cultured with Ia positive C1 or M3.1 and HEL, as measured in the HT-2 assay, indicating antigen presentation (data not shown). To determine whether tubular cells process the HEL, Ia-positive C1 and M3.1 cells were fixed with 1% paraformaldehyde after overnight exposure to HEL (Table 4). Fixed cells retain their ability to present HEL to the A2A2 T hybrid. Higher doses of fixed APC are required to obtain equal activation of A2A2 cells. Fixation of the cells prior to exposure to HEL abolishes activation of A2A2. This indicates that the tubular cells process the HEL prior to presentation in a fashion similar to classic antigen presenting cells of hematopoietic lineage (that is, macrophages and B cells).

Cytokine gene expression

Since PT share Ia expression and antigen presentation with cells of the immune system, we sought to determine whether these SV40 transformed tubular cells have other accessory

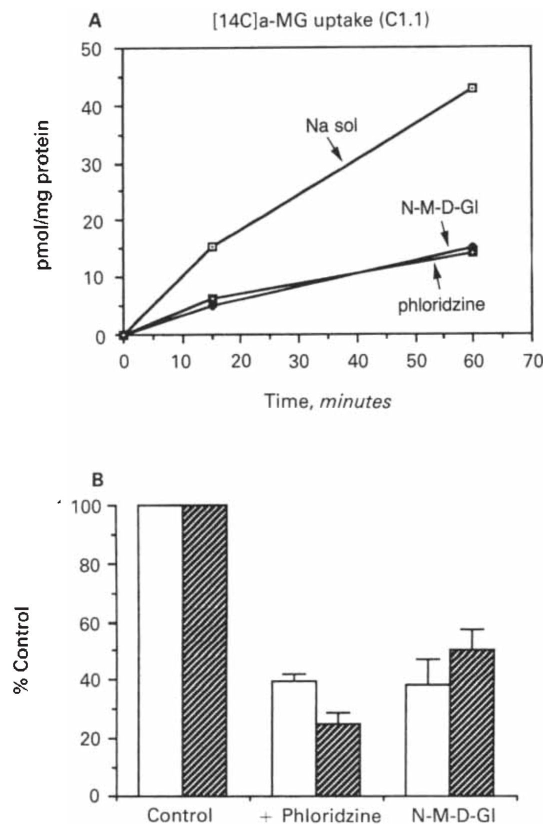

Fig. 3. *Sodium-dependent glucose transport by transformed tubular cells (C1.1 and M3.1).* Cells were grown in 6-well plates to confluence and assayed for uptake of methyl α-D-glucopyranoside [^{14}C] after 15 and 60 min. **A.** Uptake by C1.1 cells in sodium-containing solution (Na sol) with or without 0.5 mM phloridzine or in sodium-free solution (N-methyl-D-glucamine, N-M-D-Gl). Results are expressed as pmol/mg protein (duplicate determinations from a representative experiment). **B.** Inhibitory effect of 0.5 mM phloridzine or replacement of sodium with N-M-D-G1 in C1.1 (□) and M3.1 (▨), at 60 min. Results are expressed as the mean percentage of control ± SE. Data are pooled from 3 separate experiments from which Fig. 3A is an example.

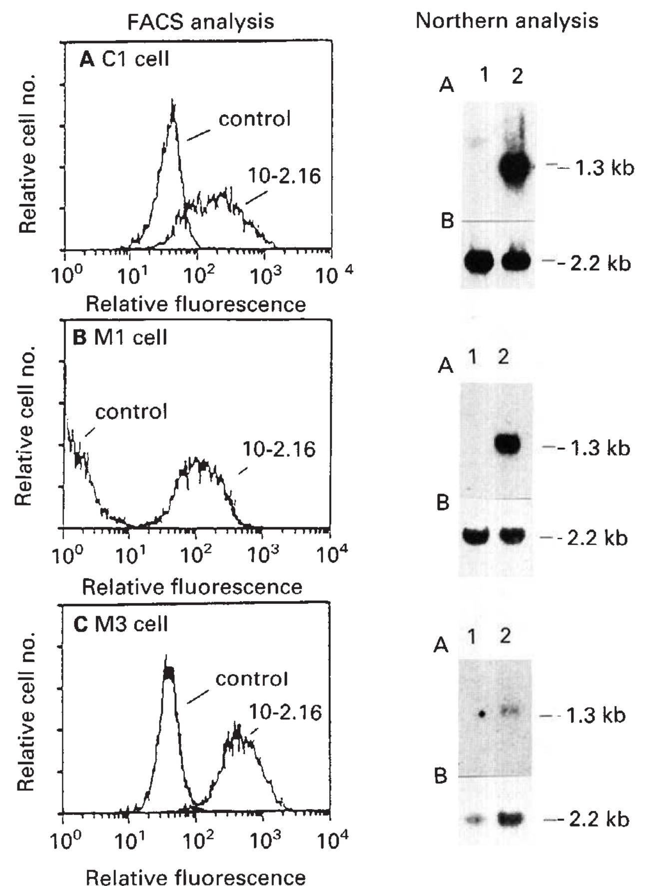

Fig. 4. *MHC class II expression by flow cytometry and Northern analysis in rIFN-γ stimulated tubular cells.* Flow cytometry (left panels): C1, M1 and M3 cells were stimulated with rIFN-γ (100 U/ml) for 72 hr and stained with MAb 10-2.16. The control represents IFN-γ stimulated cells stained with secondary step reagent only. Staining with irrelevant MAb was superimposable to controls (not shown). Northern analysis (right panels): Cells were stimulated for 24 to 48 hr with rIFN-γ. Blots were hybridized with an A_α-specific Ia probe (**A**), and also rehybridized with β-actin (**B**). Lane 1, unstimulated cells; lane 2, rIFN-γ stimulated cells (C1, 500 U/ml; M1, 1000 U/ml; M3, 100 U/ml).

functions, such as cytokine production. The cells (C1 and C1.1) were stimulated with rIL-1α, TNF-α, LPS, rIL-4 or rIFN-γ and RNA was extracted for Northern analysis. Blots were probed for IL-1α, IL-β, TNF-α or IL-6. Table 5 summarizes the results. No steady-state mRNA levels for IL-1α, IL-1β, TNF-α, and IL-6 are detected in total RNA obtained from unstimulated cells. Upon stimulation with rIFN-γ, rTNF-α, or rIL-4, transcripts for none of the above cytokines are detected. However, upon stimulation with rIL-1α, transcripts for TNF-α are now present (Fig. 7). Two different sources of murine rIL-1α (Hoffmann-La Roche and Pfizer) gave similar TNF-α mRNA induction. These cells also express TNF-α when stimulated with LPS (1 μg/ml). TNF-α mRNA is apparent within 24 hours.

Discussion

Renal allograft rejection [1], graft-versus-host disease [2] and lupus nephritis [3, 4] induce MHC class II expression in the proximal tubular epithelium of the kidney. To study the expression, regulation and function of murine Ia antigens on PT, we have established SV40 transformed clonal PT-like epithelial cell lines from normal and autoimmune MRL-*lpr* mice. These cell lines retain many differentiated PT cell functions, and also display differentiated accessory cell functions such as processing and presentation of antigen. No inherent difference in these accessory functions could be detected between C3H/FeJ and autoimmune MRL-*lpr* derived PT. Transformation with origin-defective SV40 DNA immortalizes cells in a differentiated state, providing large numbers of homogeneous clonal cells for analysis. SV40 DNA has been used to generate transformed fibroblasts [27], macrophages [28], thymic epithelial cells [29] and also recently a PT cell line [30]. A similar approach has used the *ras* oncogene to transform PT [31].

Characterization of our cell lines revealed that they have many physiological functions typical of normal proximal tubular epithelium: brush border enzymatic activity, growth in response to EGF, hormonal responsiveness to Ang II and PTH, and sodium-dependent glucose uptake. Of these, Na$^+$-dependent glucose uptake is a highly specific property [22, 32], since

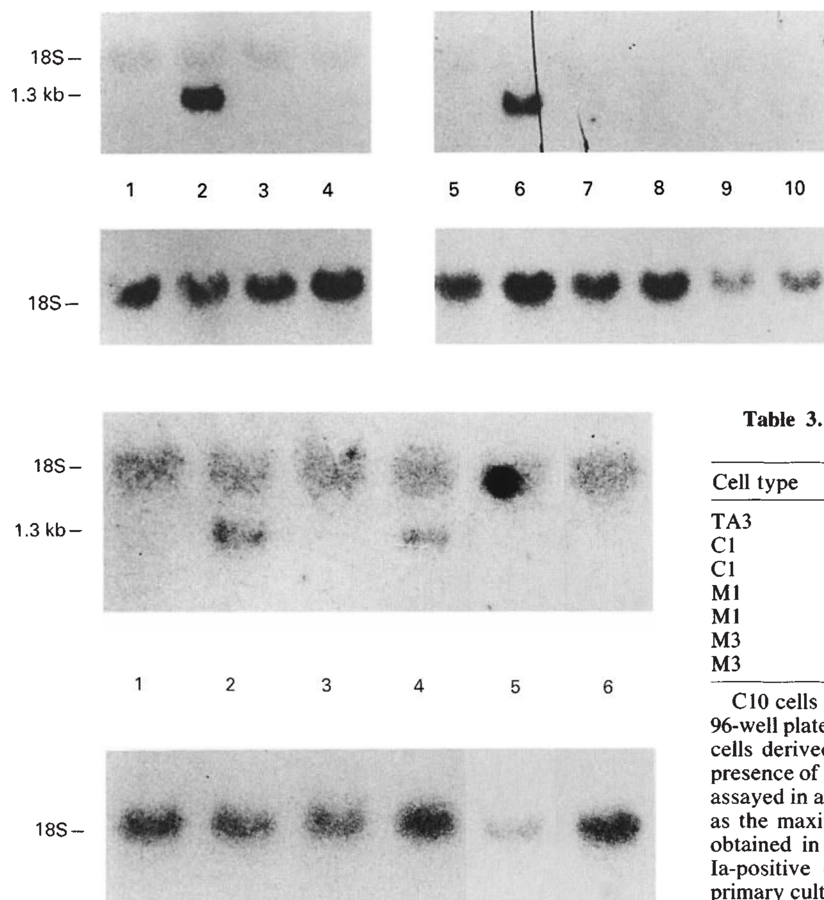

Fig. 5. *Blocking of rIFN-γ stimulated I-A_α gene expression by anti-murine IFN-γ MAb R4-6A2, cycloheximide, dexamethasone, or hydrocortisone (Northern analysis).* Cells (C1) were grown to confluence for 48 hr, and stimulated with rIFN-γ in the presence of inhibitors for 24 hr. Lane 1 and 5, control (no rIFN-γ); lane 2, rIFN-γ (100 U/ml); lane 3 and 4, rIFN-γ + R4-6A2 (0.5 and 5 μg/ml, respectively); lane 6, rIFN-γ (1000 U/ml); lane 7, rIFN-γ + cycloheximide (0.5 μg/ml); lane 8, cycloheximide (0.5 μg/ml); lane 9, rIFN-γ + hydrocortisone (10^{-4} M); lane 10, rIFN-γ + dexamethasone (10^{-4} M).

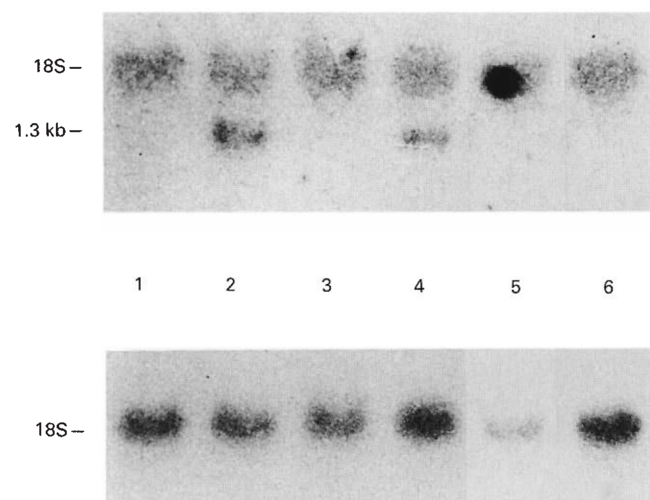

Fig. 6. *Tubular epithelial cells (C1) express Ia in response to rIFN-γ, but not rTNF-α, rIL-4, and LPS (Northern analysis).* Lane 1, unstimulated cells; lane 2, rIFN-γ (10 U/ml); lane 3, rTNF-α (100 ng/ml); lane 4, rIFN-γ (10 U/ml) + rTNF-α (100 ng/ml); lane 5, rIL-4 (1000 U/ml); lane 6, LPS (1 μg/ml).

Table 3. Ia-positive tubular epithelial cells process and present antigen to Ia^k-restricted T cell hybridomas

Cell type	IFN-γ	Ia	cpm × 10^3
TA3	–	+	299.4
C1	–	–	0.7
C1	+	+	14.4
M1	–	–	1.7
M1	+	+	34.0
M3	–	–	0.6
M3	+	+	74.2

C10 cells (HEL-specific, Ia^k-restricted; 1×10^5) were co-cultured in 96-well plates with varying numbers (3×10^3 to 10^5) of tubular epithelial cells derived from C3H/FeJ (C1) or MRL-*lpr* mice (M1, M3), in the presence of HEL (50 μg/well). The IL-2 content in the supernatants was assayed in a secondary culture, using HT-2 cells. Results are expressed as the maximum proliferative response ([^{3}H]thymidine incorporation) obtained in the secondary culture (generally achieved with 3×10^4 Ia-positive cells), using serial dilutions of the supernatants in the primary culture. Cells were checked for Ia positivity by flow cytometry using MAb 10-2.16.

it is not found on other epithelial cells in the kidney. In addition, the transformed cell lines show enhanced proliferation to EGF, a known growth factor of proximal tubules [33, 34]. This is in agreement with the detection of EGF receptors on transformed PT reported by two other groups [30, 31]. Furthermore, our cell lines display an appropriate response to Ang II (PGE_2 synthesis) and to PTH (cAMP synthesis). Unexpectedly, these cells also generate cAMP in response to AVP, a characteristic of distal or collecting duct epithelium. A cAMP response to AVP was present in subcloned cells, making it unlikely that this is the result of a contaminating distal cell type. The presence of AVP receptors is not without precedent since the proximal tubular-like pig epithelial cell line LLC-PK_1 also produces cAMP in response to AVP [35, 36]. Gene derepression by cellular transformation may account for the expression of some distal tubular epithelial functions by these lines. Taken together, the characterization of these cell lines indicates that we have generated PT-like cell lines with many differentiated functions of normal PT epithelium, providing an excellent model system.

The generation of these cell lines allowed us to study the regulation and function of tubular epithelial Ia expression. The cell lines do not constitutively express MHC class II antigens. This is in agreement with other investigators using transformed [30] or untransformed tubular cells [37] which have only minimal levels of constitutive Ia [30, 37]. We were unable to generate cell lines which constitutively express Ia, even from nephritic MRL-*lpr* mice which have Ia-positive PT in vivo [4]. Freshly isolated PT from MRL-*lpr* mice in primary culture lose Ia expression (unpublished observation). This suggests that a factor which is responsible for maintaining Ia expression in vivo is lost during the process of culture and/or transformation. A similar situation occurs during culture of murine macrophages which lose Ia expression in vitro [38].

The tubular cell lines could be readily induced to express MHC class II antigens by IFN-γ. This is in agreement with another study that found that IFN-γ induces HLA-DR expression in human renal cortical tubular cells [37]. Injection of IFN-γ is also known to induce PT Ia expression in vivo [39]. Other known Ia-inducing factors such as IL-4 and TNF did not induce class II in our cell lines. No potentiating activity of TNF on IFN-γ induced class II expression could be detected, whereas such an effect has been described in the macrophage-like cell line WEHI-3 [40]. We also demonstrate, for the first time, that IFN-γ induced Ia-expression in tubular epithelium is corticosteroid-sensitive, suggesting that a steroid-responsive element is involved for class II expression in these cells, as in B cells [41] and macrophages [42]. In contrast, other investigators have not demonstrated inhibition of IFN-γ induced Ia antigens

Table 4. Processing of HEL by tubular epithelial cells

Cell type	IFN-γ	Ia	APC treatment: Fixation	APC treatment: HEL pre-fixation	APC treatment: HEL during co-culture	IL-2 *(U/ml)* APC 10^4	IL-2 *(U/ml)* APC 3×10^4
2B1	−	+	−	−	+	>160	>160
	−	+	+	+	−	>160	>160
	−	+	+	−	+	20	80
C1	−	−	−	−	+	<20	<20
	+	+	−	−	+	>160	>160
	+	+	+	+	−	80	>160
	+	+	+	−	+	<20	<20
M3.1	−	−	−	−	+	<20	<20
	+	+	−	−	+	>160	>160
	+	+	+	+	−	80	>160
	+	+	+	−	+	<20	<20

Varying numbers of rIFN-γ stimulated tubular or control 2B1 cells (3×10^3 to 10^5 antigen presenting cells, APC) were co-cultured with 10^5 HEL-specific Ia^k-restricted A2A2 cells for 24 hours. HEL (50 μg/well) was added to all wells except those that had been pulsed with HEL overnight prior to fixation with 1% paraformaldehyde (PF). Supernatants were assayed for IL-2 content in the HT-2 assay. Results are expressed in arbitrary U/ml of IL-2 [26]. Two doses of APC are shown (10^4 and 3×10^4 APC). Ia expression was determined by flow cytometry.

Table 5. Cytokine profile of SV40 transformed tubular epithelial cells

Stimuli	Dose	Cytokine mRNA: IL-1α	IL-1β	TNF-α	IL-6
None	−	−	−	−	−
rIL-1α	0.1–1 μg/ml	−	−	+	−
rTNF-α	100 ng/ml	−	−	−	−
LPS	1–5 μg/ml	−	−	+	−
rIL-4	1000 U/ml	−	−	−	ND
rIFN-γ	10–1000 U/ml	−	−	−	−

Tubular epithelial cells (C1) were grown for 24 to 48 hours to confluence before stimulation (24 to 48 hr) with specific cytokines. RNA was extracted, electrophoresed and blotted as described in **Methods**. Blots were probed with ^{32}P-labeled (random priming) specific cDNA probes, and exposed for 5 to 10 days to Kodak X-AR film. On each blot, positive controls were included (RNA extracted from macrophage 59 or WEHI-3 cells). ND, not done.

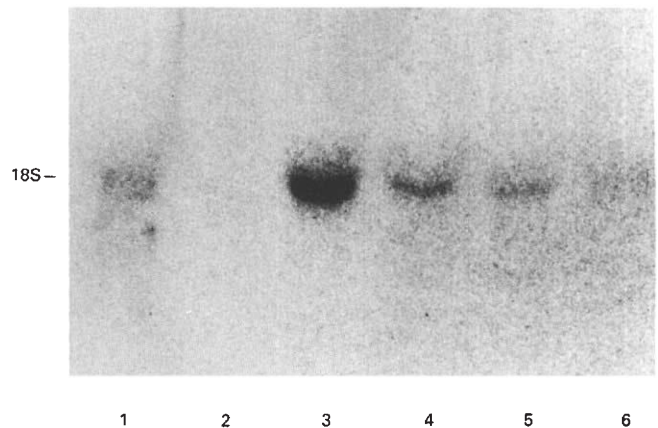

Fig. 7. *TNF-α gene expression by Northern analysis in rIL-1α and LPS-stimulated cells (C1).* Lane 1, positive control (MRL-*lpr* kidney cortical RNA); lane 2, unstimulated cells; lane 3, rIL-1α (Hoffmann-La Roche, 100 ng/ml, 24 hr); lane 4, rIL-1α (Hoffmann-La Roche, 100 ng/ml, 48 hr); lane 5, rIL-1α (Pfizer, 100 ng/ml, 48 hr); lane 6, LPS (1 μg/ml, 48 hr).

by corticosteroids in human cortical tubular cell cultures [37, 43]. This discrepancy might be explained by contaminating cells or species difference [43].

The regulation of IFN-γ induced Ia expression has been studied in both immune cells of hematopoietic lineage [40, 42, 44, 45] and non-hematopoietic cell lines such as HeLa cells [46], fibroblasts [47], glioblastoma multiforme cells [48] and osteosarcoma cells [49]. However, the regulation of tubular epithelial Ia gene expression has not been previously studied. In our cell lines, IFN-γ mediated Ia-induction was highly cycloheximide-sensitive, indicating that protein synthesis is necessary to promote Ia expression in these cells. Ia induction by IFN-γ in a glioblastoma cell line is not cycloheximide-sensitive [48], whereas such induction in macrophage-like P388D1 cells [44] and osteosarcoma cells [49] is cycloheximide-sensitive. IFN-γ induced MHC class II gene expression in HeLa cells [46], glioblastoma cells [48] and class II transfected fibroblasts [47] is regulated by transcriptional control sequences and nuclear factors which bind to conserved sequences in the 5′ upstream untranslated region. In P388D1 cells, IFN-γ induces DNA-specific binding proteins which bind to conserved sequences in the I-Eβ promoter region [45]. It will be of interest to determine whether the transcriptional regulation of Ia expression by IFN-γ in tubular epithelial cells is different from that in immune system cells.

MHC class II molecules are polymorphic cell surface heterodimers crucial for foreign antigen presentation to T cells of the $CD4^+$ phenotype [50]. Macrophages and B cells are the classical antigen presenting cells (APC). We now report, for the first time, that tubular epithelial cells are capable of MHC-restricted, foreign protein-specific antigen presentation. Other non-hematopoietic cells such as thyroid epithelial cells [51, 52], gut epithelial cells [6] and chondrocytes [5] are able to present antigen in the context of class II, while other cells such as keratinocytes are incapable of this function [7]. In another study [30], PT derived from an interstitial nephritis model can support the proliferation of a T cell clone specific for the nephritic antigen produced by these PT, confirming that PT can interact with cells of the immune system to promote inflamma-

tion. On the other hand, cultured human kidney epithelial cells induce only limited proliferation of allogeneic lymphocytes in a primary mixed lymphocyte kidney culture when compared with peripheral blood mononuclear stimulator cells [53]. Although PT are less efficient APC than B cell lines, their abundance in the kidney could contribute substantially to antigen presentation.

Various cells of non-hematopoietic lineage express cytokines and can influence immune-mediated cellular injury. Endothelial and vascular smooth muscle cells for example can be induced to express the cytokines IL-1 and TNF [54–56]. Other investigators demonstrated IL-1 production by keratinocytes [57], and mesangial cells can produce IL-1 [58, 59] and TNF [60]. In a preliminary report, others have also shown an "IL-1 like" bioactivity in supernatants from cultured PT [61]. We investigated whether tubular epithelial cell lines can express cytokines by measuring mRNA for cytokines. Of the four cytokines tested, IL-1α, IL-1β and IL-6 are not expressed, whereas upon stimulation with IL-1α or LPS, tubular epithelial cells express TNF-α mRNA. The precise role of TNF expression by tubular epithelial cells and other non-immunologic cells is largely unknown. However, TNF has many proinflammatory effects such as prostaglandin release, collagenase synthesis, cytotoxicity and macrophage chemoattraction [62]. We have previously shown increased IL-1 and TNF production in the kidneys of MRL-*lpr* mice with nephritis [15, 16], suggesting a crucial role of these cytokines in the pathogenesis of autoimmune renal injury. We speculate that local release of TNF by PT may have a pathogenic role in immune renal injury.

In conclusion, transformation of renal cortical tubular cells resulted in the generation of differentiated PT-like epithelial cell lines. These cell lines can express Ia antigens when induced with IFN-γ, and Ia expression confers antigen presenting ability to these cells. Furthermore, these cell lines express TNF-α when stimulated with IL-1 or LPS. These results suggest that in addition to their physiological role of fluid and solute reabsorption, PT can participate in immune responses in the kidney.

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