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Complement C3 gene expression and regulation in human glomerular epithelial cells

S. H. SACKS, W. ZHOU, A. PANI, R. D. CAMPBELL* & J. MARTIN† *Renal Laboratory, United Medical and Dental Schools, Guy's Campus, London, *MRC Immunochemistry Unit, University of Oxford and †Institute of Nephrology, Cardiff Royal Infirmary, Cardiff*

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SUMMARY

Extra-hepatic synthesis of complement is thought to mediate local tissue inflammatory injury. To investigate this phenomenon in the glomerular epithelial cell (GEC), we examined the biosynthesis and regulation of gene expression of the third component of complement in isolated human GEC derived from normal tissue. Metabolic labelling and immunoprecipitation studies demonstrated that C3 protein was synthesized, processed and secreted by GEC under basal conditions. The secreted C3 α and β polypeptide chains had identical electrophoretic mobilities with those of hepatic C3. Examination of cellular RNA using semi-quantitative polymerase chain reaction (PCR) showed that C3 gene expression was present in unstimulated GEC and was increased by stimulation with interferon-γ (IFN-γ) in a time- and dose-dependent manner. Tumour necrosis factor-α (TNF-α), while mediating an increase in monocyte U937 C3 expression, revealed no evidence of regulation of GEC C3 gene expression. These results indicate that human GEC spontaneously express the C3 gene and that increased gene expression is regulated by IFN-γ. These observations may reflect part of a wider mechanism of protection against or mediation of local, immune-mediated tissue injury.

INTRODUCTION

The third component of complement is the most abundant complement protein in the serum. It has important pro-inflammatory and immunostimulant effects and plays a crucial role in the defence against foreign pathogens.[1] At the same time, C3 may protect against immune complex injury since C3 increases the solubility of immune complexes[2] and hereditary deficiency of C3 predisposes to immune complex disease, particularly affecting the kidney.[3]

The main site of C3 synthesis is the liver[4] but extrahepatic synthesis has been described in a number of cells including macrophages and monocytes,[5-7] skin and synovial cells,[8,9] endothelial cells[10] and neutrophils.[11] Extrahepatic synthesis is increased in a number of inflammatory conditions and can be regulated by inflammatory cytokines.[12] In the kidney, C3, C2, C4 and Factor B gene expression have been found in mice with systemic lupus erythematosus (SLE), where it was thought that local tissue complement gene expression could contribute to the pathogenesis of nephritis.[13] More recently, C4 gene transcription has been reported in human kidney[14] and C3 transcription in proximal renal tubule cells.[15]

The glomerular epithelial cell (GEC) is a specialized cell which forms part of the glomerular filtration barrier and is a target for complement-mediated damage in a number of types of immune complex glomerulonephritis.[16] Evidence that the GEC is more actively involved in the handling and possibly disposal of immune complexes derives from the presence of cell-surface receptors for C3b[17] and the demonstration of cytoplasmic and intravesicular Ig and C3[18] in human GEC. The present study set out to determine if cultured GEC also synthesize C3 and whether, using semi-quantitative polymerase chain reaction (PCR), C3 gene expression is regulated by inflammatory cytokines.

Correspondence: Dr S. Sacks, 3rd Floor Guy's Tower, Guy's Hospital, St Thomas Street, London SE1 9RT, U.K.

MATERIALS AND METHODS

Cell culture

Primary GEC cultures were derived from normal renal cortex as previously described,[19,20] using nephrectomy specimens with well circumscribed tumour in the opposite pole. In all, four such preparations were performed and the results described here were derived from experiments using all GEC preparations. Cell monolayers were removed using trypsin–EDTA and grown in RPMI-1640 with 10% foetal calf serum (FCS), 5 μg/ml insulin, 5 μg/ml transferrin, 0·005 μg/ml sodium selenite, 0·4 μg/ml hydrocortisone, 1 mM sodium pyruvate, 15 mM HEPES, and 0·09% $NaHCO_3$, and used for study no later than the fourth passage. GEC were characterized by their morphological appearance[21] and by positive staining of the cytoskeleton with

anti-cytokeratin (Sigma Chemical Co., Poole, U.K.; 1/800) (excluding glomerular mesangial cells), strong positive staining with anti-vimentin (a marker for visceral GEC) and negative staining with anti-factor VIII (Dako, Glostrup, Denmark; 1/5) (a marker for endothelial cells), by indirect immunofluorescence.[20] Staining was absent for non-specific esterase[22] (a macrophage marker). HepG2 hepatoma cells were grown in 10% FCS–DMEM (Dulbecco's minimal essential medium) containing glutamine and penicillin/streptomycin. U937 human monocytic cells were from the European Collection of Animal Cell Cultures (Salisbury, U.K.). Fresh human peripheral blood lymphocytes (PBL) and Epstein–Barr virus (EBV)-induced B-lymphoblastoid cell lines were used as negative mRNA controls.

Confluent GEC monolayers were cultured for 12 hr in serum-free medium, split into 1×10^5 cells/flask, and cultured for a further 24 hr in serum-free medium or medium supplemented with 1000 U/ml interferon-γ (IFN-γ) (Sigma) or 100 ng/ml tumour necrosis factor-α (TNF-α) (Sigma). RNA was then isolated for cDNA–PCR analysis. For metabolic labelling, cells were cultured with control or IFN-γ medium for 12 hr before the addition of radiolabelled methionine.

PCR amplification

Total cellular RNA was extracted from tissue culture cells by single-step guanidium thiocyanate–phenol–chloroform extraction as described previously.[23] The product of 3×10^5 cells was finally suspended in 10 μl dH_2O and RNA yield was measured as described previously.[24] RNA extraction from solid tissue was carried out as above using 3–5-mm^3 fragments of renal cortex snap frozen in liquid nitrogen and disrupted in micro-homogenizer tubes (Biomedix, Pinner, U.K.). cDNA synthesis was carried out with 5 μg total cellular RNA and 160 ng oligo$(dT)_{12-18}$ primer (Pharmacia, Milton Keynes, U.K.) in a 20-μl solution with 50 mM Tris–HCl pH 8·3, 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 1·5 U/μl RNasin (Promega, Madison, WI), 500 μM each dNTP (Pharmacia), and 200 U Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Uxbridge, U.K.). After 40 min at 37° the incubation was repeated with a further 200 U reverse transcriptase. cDNA was stored at −20° until use.

PCR[25] was carried out with cDNA diluted to reflect 0·15 μg RNA, 3 U Taq polymerase (Promega) and 12·5 pmol each of 5′ and 3′ oligonucleotide primers in 25 μl of a solution containing 10 mM Tris–HCl pH 9, 50 mM KCl, 2 mM MgCl, 0·01% gelatin w/v, 0·1% Triton X-100, 200 μM each dNTP. Each cycle of PCR included 1 min of denaturation at 94°, 1 min of primer annealing at 65°, and 3 min of extension/synthesis at 72°. After the last cycle, samples were incubated for a further 10 min at 72°. PCR products (25 μl) were separated on 1·2% agarose gels and stained with ethidium bromide. DNA sizing markers [1 kilobase (kb) and 123 base pair (bp)] (Gibco) were run with each gel. PCR was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus, Buckinghamshire, U.K.) found to provide a uniform temperature independent of the position of the sample tube in the block. Control PCR reactions were carried out as described in Results.

For quantification, 2 μCi [^{32}P]dCTP (Amersham International, Aylesbury, U.K.) and 12·5 pmol each of 5′ and 3′ β-actin (internal control) primers were added to each PCR mixture. Amplification of the specific and β-actin sequences was performed in the same tube as the results were identical to the specific and β-actin fragments when amplified in separate tubes (data not shown). Five microlitre PCR products were separated on 3% 3:1 NuSieve agarose gels. Gels were fixed with 7% trichloroacetic acid (TCA) and dried and exposed to X-ray films for 1 hr. Samples from a single experiment were treated simultaneously and exposed to the same autoradiographic film. The bands were scanned with a BioRad 620 video densitometer (BioRad Labs, Hemel Hempstead, U.K.) and analysed using one-dimensional analyst software. This gave consistent measurement of incorporation of radioactivity as assessed in preliminary studies by scintillation counting of the bands cut out from the gel. Results were expressed as ratios of the intensity of the band of the investigated transcript to the intensity of the β-actin band used as a standard (normalized PCR yield). All experiments were performed in duplicate and the experiments were repeated on at least two occasions. Data points are reported as means ± SD. Statistical significance was assessed using Student's *t*-test or the Binomial test as appropriate.

Quantification was mostly performed at 24 cycles since at this level of amplification the yield of PCR products was in the linear range (see Results). The method was validated using as target substrate (1) cellular cDNA diluted to reflect 0·05–0·5 μg RNA and (2) an increasing amount (1–10^4 copies/cell) of cloned cDNA (plasmid insert) added to 0·15 μg of cellular cDNA. In such experiments the yield of PCR products was linear over the range of added template (unpublished observations).

Primer specificity

Primers were designed using Oligo software (National Biosciences Inc., Plymouth, CA)[26] and synthesized using an Applied Biosystems DNA synthesizer (model 281; Cheshire, U.K.). Primer yield and quality were tested by ultraviolet (UV) spectroscopy and acrylamide gel electrophoresis. The C3–1 primer, 5′-GCT GCT CCT GCT ACT AAC CCA-3′, corresponds to positions 87–107,[27] and the C3–2 primer, 5′-AAA GGC AGT TCC CTC CAC TTT-3′, is complementary to positions 850–870. The β-actin-1 primer, 5′-ATG ATG ATA TCG CCG CGC TC-3′, corresponds to positions 46–65,[28] and the β-actin-2 primer, 5′-GCG CTC GGT GAG GAT CTT CA-3′ is complementary to positions 610–629. The specificity of the C3 PCR product was assessed by restriction fragment analysis using Nla IV restriction enzyme. The PCR band was cut from the gel, eluted by microcentrifugation and used as a template in a second PCR reaction with the C3 primers. Fifteen microlitres of the resulting product was digested with 2 U of Nla IV and the digest was electrophoresed on 1·4% agarose, using 123 bp markers. The C3 digest had two clear fragments (> 100 bp) which corresponded with the predicted sizes of 313 and 284 bp from the known cutting sequence of Nla IV (5′-GGN NCC-3′) and the genetic code for C3[27] (data not shown). Further, the C3 PCR fragment was analysed by Southern blotting using published methods.[24] The C3 fragment hybridized specifically with C3 probe, which was the 1000 bp *Bst*EII fragment of the probe pC3.11 (a gift from Dr B. Morely),[27] but not with C4 probe, which was the 476 bp *Bam*HI/KPNI fragment of the full-length probe pAT-A[29] (data not shown). These data confirm that the sequence amplified is that of C3.

Metabolic labelling and immunoprecipitation

Confluent cells were incubated with [^{35}S]methionine (500 μCi/ml) for 1 hr at 37° followed by excess cold methionine for 0–8 hr and lysed in 1% Triton X-100, 0·5% deoxycholic acid, as described previously.[30] Cell supernatants and lysates were

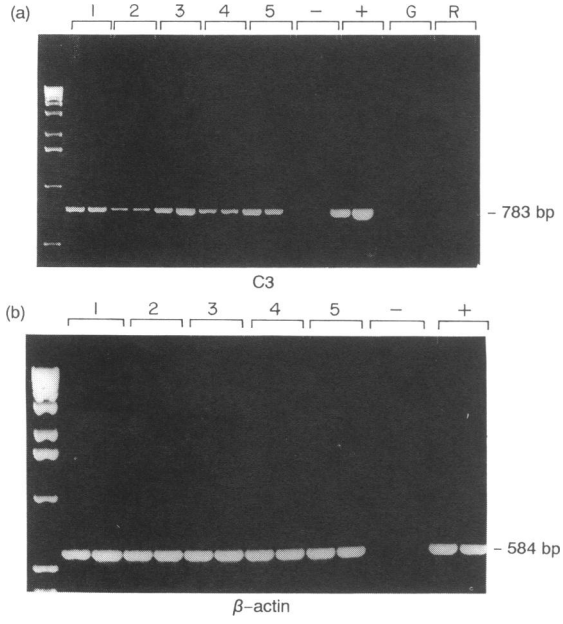

Figure 1. Detection of C3 mRNA (a) and β-actin mRNA (b) in normal renal cortex by cDNA–PCR analysis. Ethidium-stained agarose gels showing C3 and β-actin PCR fragments at 30 cycles of amplification. (1–5) Duplicate experiments with PCR substrate from five tissue donors. Control PCR reactions: (–) water replacing cDNA as PCR substrate; (+) HepG2 cDNA as substrate; (G) genomic DNA as substrate; (R) non-reverse-transcribed RNA. Shown in the first lane of each gel, a ladder of 1 kb molecular size markers. Apparent sizes of PCR fragments shown in base pairs at the right.

incubated overnight at 4° with 25 μl of anti-C3 (Incstar, Wokingham, U.K.) in a final volume of 500 μl lysate and then immune complexes were precipitated with formalin-fixed *Staphylococcus* A (Sigma), as described previously.[31] Immune complexes were dissociated by boiling in sample buffer and analysed by 7·5% or 10% SDS–PAGE in reducing conditions, using ^{14}C-methylated, 200,000, 92,500, 68,000, 46,000, 30,000, and 17,000 MW markers (Amersham).[31] Gels were impregnated with EN^{3}HANCE™ (Du Pont, U.K.) and dried and exposed to XAR X-ray film.

RESULTS

C3 gene expression in normal kidney

C3 gene transcripts were first examined in normal renal cortex. The results of cDNA–PCR analysis in five surgical specimens are shown in Fig. 1. A 783 bp product corresponding to C3 mRNA was detected with these tissues (Fig. 1). A fragment of identical size corresponding to the C3 sequence was detected in HepG2 cells, but none was detected in the negative tissue controls (not shown) or in reactions using as substrate water or non-reverse transcribed RNA (Fig. 1). No C3 fragment was detected using genomic DNA as template (Fig. 1) because the genomic target seqence contains three introns and the efficiency of PCR amplification varies inversely with the length of the target sequence.[32] These data show that the C3 PCR fragment is specific for mRNA in the target substrate and is not due to amplification from contaminating DNA.

C3 gene expression in isolated human GEC

GEC were isolated by primary culture from normal renal tissue and confluent monolayers of third to fourth passage GEC were incubated with serum-free control medium or medium supplemented with recombinant human IFN-γ. Total cellular RNA was isolated from these monolayers and examined by PCR. A 783 bp C3 product was identified in all GEC preparations, corresponding in size to the C3 fragment from HepG2. These data indicate that early passages of GEC at steady state express C3 mRNA.

Up-regulation of GEC C3 gene expression by IFN-γ

To examine the influence of cytokine on basal C3 mRNA expression, we employed conditions under which PCR amplification proceeded at an exponential rate. Under these conditions the yield of PCR product reflects the initial amount of PCR substrate.[33] The yields of C3 and β-actin (internal control) amplification products over a range of PCR cycles is shown in Fig. 2A. Above 32 cycles (data not shown) the yield of PCR products approaches saturation. Below this level of amplification, however, the yield of PCR product is in the linear range (Fig. 2A). Under these conditions the level of C3 mRNA in IFN-γ-treated cells and untreated control cells can be clearly distinguished ($P < 0{\cdot}001$). No increase in the yield of β-actin product was identified (Fig. 2A) suggesting there was no generalized increase of mRNA in IFN-γ-activated cells. The efficiency of amplification of the β-actin fragment was similar to that with the C3 fragment, which meant that the ratio of C3/β-actin products at 24 cycles of amplification could be used to compare the relative amounts of C3 mRNA in different preparations (Fig. 2B).

Measurement of the normalized yield of C3 product was used to examine the dose and time responses to stimulation with IFN-γ (Fig. 3). There was a graded increase in C3 PCR product with IFN-γ at concentrations between 10 and 1000 U/ml. The data indicate that the effect of IFN-γ on C3 gene transcription is detectable 6–24 hr after stimulation.

Influence of TNF-α on C3 gene expression

Experiments were carried out to compare the influence of inflammatory cytokines on GEC and U937 C3 gene expression. The cells were incubated for 24 hr with control medium or medium which contained 1000 U/ml IFN-γ or 100 ng/ml TNF-α. RNA was then extracted and examined by semi-quantitative PCR at 20, 24, 28 and 32 cycles of amplification. All studies were performed in parallel. Normalized yields of the C3 fragment at exponential amplification are shown in Table 1. Prolonged stimulation with TNF-α produced no detectable effect on GEC C3 gene expression, in contrast to the effect of TNF-α on U937 cells. IFN-γ led to up-regulation of C3 in both cell types. This could imply that C3 gene transcription in GEC and U937 cells are controlled by different regulatory mechanisms.

C3 protein biosynthesis by human GEC

Metabolic labelling and immunoprecipitation studies were performed to determine if the same cells which express C3 mRNA can synthesize C3 protein. The extracellular C3 was characterized by bands of ~112,000 and 72,000 MW corres-

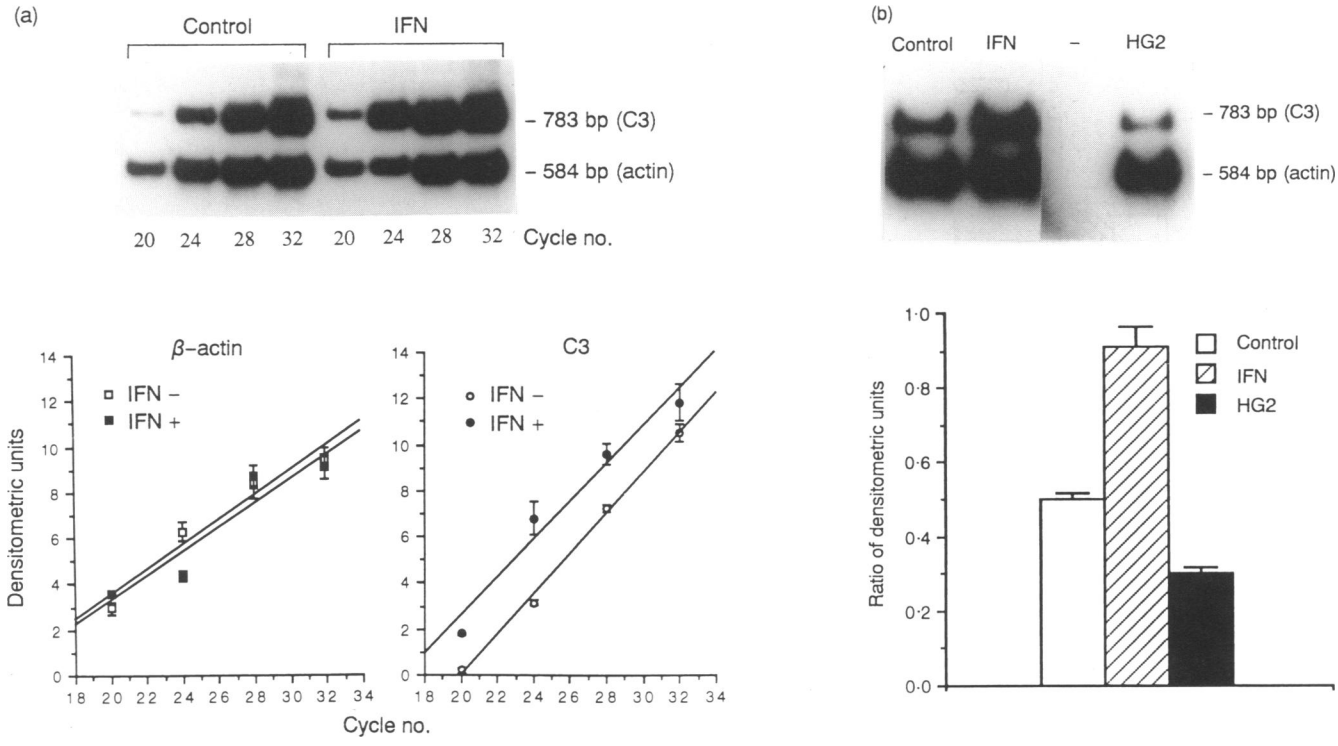

Figure 2. Relative quantification of GEC C3 gene transcripts. (a) Autoradiograph showing the C3 and β-actin PCR fragments after different numbers of amplification cycles in the presence of [^{32}P]dCTP. (lower panel) Yield of PCR fragments in arbitrary densitometric units as a function of the number of PCR cycles. Data points represent the mean of duplicate experiments. (IFN +) and (IFN −) IFN-γ-treated and untreated cells. The two data sets on the left (β-actin) do not significantly differ ($P = 0{\cdot}5$) while those on the right (C3) differ at $P < 0{\cdot}001$. (b) Bar chart showing normalized yields of C3 fragment at 24 cycles of PCR in densitometric units. Data points represent the means of four experiments. Autoradiograph showing representitive PCR products. (Control) Untreated GEC; (IFN) IFN-γ-treated GEC; (−) water replacing cDNA as PCR substrate; (HG2) unstimulated HepG2 cDNA as substrate. Normalized C3 PCR yields in stimulated and unstimulated GEC differ at $P < 0{\cdot}05$.

ponding to the α- and β-polypeptide chains.[34] These were detected in both the culture fluid of control- and IFN-γ-stimulated GEC (Fig. 4). Corresponding bands were precipitated from the culture fluid of HepG2. Quantitative assessment of secreted C3 was not undertaken, but the intensities of the α- and β-bands in the culture supernatant indicate that IFN-γ leads to increased secretion of C3.

Pulse–chase studies showed that newly labelled C3 was detectable in the GEC culture fluid 1 hr after initial exposure to labelling medium. Secreted, labelled C3 increased in amount for at least 6 hr (Fig. 5). In addition, GEC contained a major reactive band of ~185,000 precipitated with anti-C3 (Fig. 5). This band probably corresponds to pro-C3,[8] although higher resolution gels are needed to confirm this. As the pulse–chase interval increased there was a reduction in the labelled, putative pro-chain intracellularly corresponding to an increase in the intensity of the α- and β-chains in the extracellular fluid. This is consistent with metabolism of the 185,000 MW chain and secretion as α- and β-chains.

DISCUSSION

Previous studies have shown that C2, C3, C4 and Factor B genes are expressed in murine kidney[13] and C4 gene in human kidney.[14] The present study shows that the human C3 gene is transcribed in normal renal cortex. It was unclear from these earlier studies whether C3 was synthesized by native renal cells or by locally invasive cells such as macrophages or monocytes. Brooimans *et al.*[15] reported that C3 was synthesized by renal tubule cells. Witte *et al.*[35] localized C4 transcripts to the proximal renal tubule using *in situ* hybridization. The results of these current experiments clearly demonstrate that C3 proteins are synthesized and secreted by isolated glomerular epithelial cells.

The presence of a local source of C3 has implications for the normal and pathological functions of complement at this site. Local tissue production of C3 could offer a kinetic advantage in that most foreign organisms enter the body by local tissue invasion before entering the vascular compartment. Activation of C3 by the classical or alternative pathways could help to eliminate the offending organism by opsonization and lysis at the portal of entry.

An important mechanism of nephritis derives from the interaction between antigen, antibody and complement at the interface between the glomerular epithelium and capillary wall.[36,37] Activation of C3 can lead to reduced formation and increased dissolution of immune complexes;[2] on the other hand, activation of complement generates vasoactive and chemotactic peptides that lead to recruitment from the intravascular compartment of the cellular and soluble mediators of inflammation.[1] Local synthesis of complement could therefore significantly modify the evolution of glomerulonephritis either by protection against or mediation of immune injury.

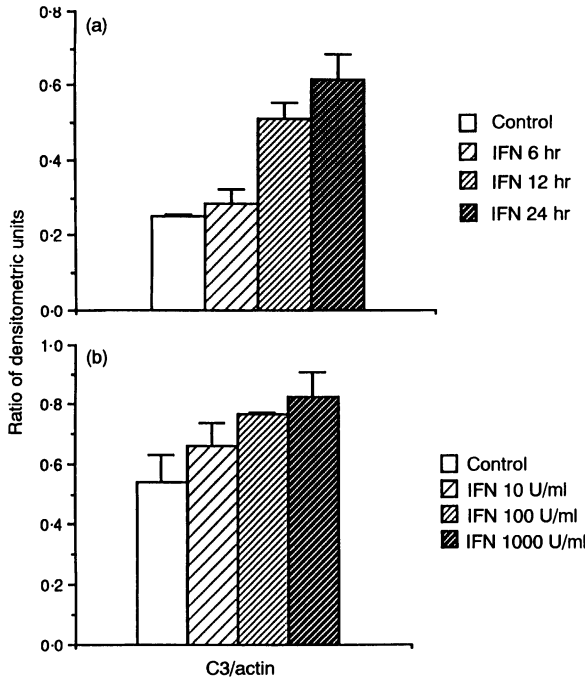

Figure 3 Dose and time effects of IFN-γ on GEC C3 gene expression. (a) Normalized yields of C3 PCR products at 24 cycles of amplification in cells stimulated with 1000 U/ml IFN-γ for increasing time intervals, as shown. (b) Normalized yields of C3 products in cells stimulated for 12 hr with increasing concentrations of IFN-γ.

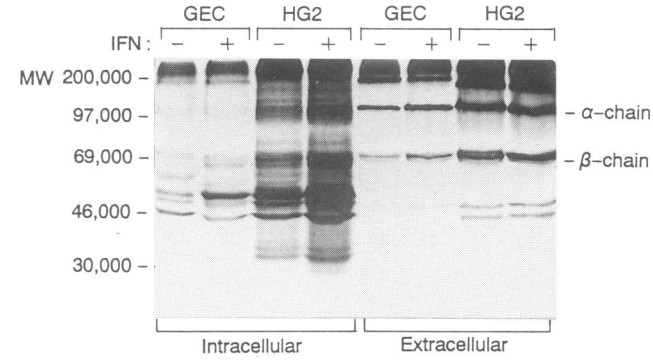

Figure 4. C3 immunoprecipitates from the intracellular and extracellular compartments of GEC and HG2 cells analysed by 10% SDS–PAGE under reducing conditions. Cells were incubated with [^{35}S]methionine for 1 hr and cold methionine for 8 hr before precipitation with anti-C3. In the experiment shown lysates were not pre-absorbed with *Staphylococcus* A and autoradiographs were over-exposed to show the presence of C3 products in the culture supernatant. (IFN +) or (−), pretreatment or not with IFN-γ.

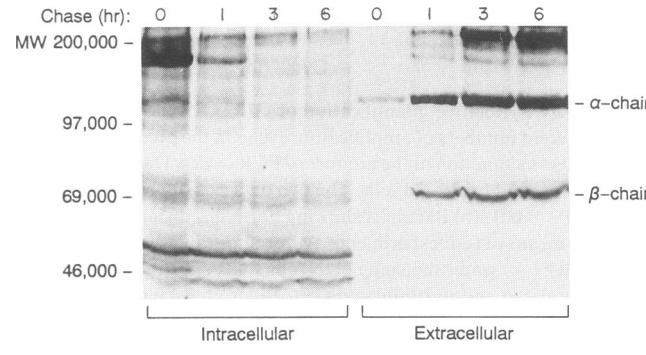

Figure 5. Pulse–chase experiment showing the kinetics of the synthesis and secretion of C3 polypeptide chains by GEC. Cells were labelled and then incubated for an increasing period of time with cold methionine. Autoradiograph showing C3 immunoprecipitates at different times (hr) analysed by 7·5% SDS–PAGE in reducing conditions.

Table 1. Normalized yield of C3 PCR products in TNF-α-treated-, IFN-γ-treated and untreated control cells. Data shown, calculated for products in the linear range of PCR amplification (24 cycles with GEC; 28 cycles with U937 cells). *P*-values shown for differences between stimulated and unstimulated cells of the same type

	Control	IFN-γ (1000 U/ml)	TNF-α (100 ng/ml)
GEC	0·502 ± 0·054	1·73 ± 0·042 (P = 0·002)	0·486 ± 0·033 (P = NS)
U937	0·005 ± 0·004	0·038 ± 0·011 (P = 0·05)	0·233 ± 0·025 (P = 0·006)

The results of these current studies also suggest that the regulation of C3 gene expression in GEC differs from that in other complement-producing cells. The effects of IFN-γ appeared to be non-specific in that C3 gene expression was shown to be increased in previous studies with monocytes[7] and hepatocytes[12] and in the present study with both GEC and monocytic cells. However, TNF-α appeared to exert a specific influence on monocytic cells, with no evidence of increased C3 gene transcription in GEC. Botto *et al.*[11] reported that 5 ng/ml of TNF-α was sufficient to induce C3 protein synthesis in neutrophils, with a clear increase in C3 mRNA at 8–24 hr after stimulation. However, in the present series of experiments even with prolonged stimulation and using a relatively high dose of TNF no effect on GEC C3 gene expression was observed. It is inferred from these results that C3 gene transcription in human GEC and monocytes are regulated by different molecular pathways. Alternatively, it is possible that GEC lack receptors for TNF-α, but this seems unlikely since there are numerous reports of different biological responses in GEC mediated by TNF-α.[38]

The present study was greatly facilitated by the use of the PCR. It allowed early glomerular cell cultures to be used sparingly for gene expression studies so that biosynthesis of complement protein could be examined in the same early passages. Later cultures of glomerular cells are reported to undergo de-differentiation[39] and the present approach meant that this potential difficulty was avoided. Furthermore, although the amplification of nucleic acid provides an indirect method of quantification (of gene expression) and is subject to experimental artefact, the method here provided a consistent level of discrimination in activated and non-activated cells, using as a background an internal control transcript with similar amplification characteristics to the investigated transcript. A

similar approach was used by Noonan *et al.*[33] to determine the expression of multi-drug resistance gene in human tumours. These workers reported close agreement between the results obtained by PCR and those by independent measurement of specific mRNA by filter hybridization.[33]

In conclusion, our data suggest that C3 mRNA is spontaneously expressed and translated into protein in human glomerular epithelial cells. IFN-γ, but not TNF-α, increased the expression of C3 mRNA, distinguishing GEC from other non-hepatic complement-producing cells. GEC possess complement receptors[17] and inhibitors[40,41] which could interact with locally produced (or circulating) complement and could influence the lifetime of glomerular immune complexes. In addition, local synthesis of C3, perhaps driven by IFN-γ or other local inflammatory mediators, could possibly contribute to the genesis of tissue injury at this site.

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