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C1q, a subunit of the first component of complement, enhances antibody-mediated apoptosis of cultured rat glomerular mesangial cells

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SUMMARY

We have shown previously that IgG2a anti-Thy-1 MoAb (ER4G) induces apoptosis of rat mesangial cells (GMC) *in vitro*. Since the classical complement pathway plays an essential role in Thy-1 nephritis, we analysed whether C1q, a subunit of the first component of complement, enhances the ER4G-mediated apoptosis of rat GMC. Two different subclasses of anti-Thy-1 MoAb, ER4G (IgG2a) and ER14 (IgG1), were used. It was established that ER4G binds C1q efficiently, while ER14 reacts poorly with C1q. For the experiments of apoptosis, quiescent rat GMC were exposed for 1 h at 37°C to a fixed concentration of anti-Thy-1 MoAb and incubated further for 16 h at 37°C in the presence or absence of C1q. GMC exposed to medium (M-GMC) followed by incubation of the cells with medium alone was used as controls. Apoptosis was assessed by morphological studies and quantitative analysis on FACS using FITC-annexin V (the annexin V methods) or bicolour FACS analysis using FITC-annexin V and propidium iodide (the annexin V/PI method). With the annexin V method, M-GMC revealed 9·4 ± 1·4% apoptosis. C1q had only marginal effects on apoptosis of M-GMC. GMC exposed to ER4G (ER4G-GMC) and further incubated with medium in the absence of C1q resulted in 25·7 ± 5·7% apoptosis ($P < 0.01$ relative to control). Incubation of ER4G-GMC together with 100 μg/ml of C1q significantly increased GMC-apoptosis up to 39·4 ± 4·9% ($P < 0·01$ relative to ER4G-GMC incubated in the absence of C1q). This enhancing effect of C1q on apoptosis of ER4G-GMC was time- and dose-dependent. In contrast, C1q did not significantly alter the apoptosis of either GMC exposed to ER14 (ER14-GMC) or to $F(ab')_2$-ER4G ($F(ab')_2$-ER4G-GMC), while ER14-GMC or $F(ab')_2$-ER4G-GMC incubated with medium resulted in significant apoptosis compared with control. These results were supported by morphological studies and bicolour FACS analyses in time course experiments using the annexin V/PI method. The effect of C1q is dependent on the presence of intact C1q-containing globular heads and does not occur with collagen-like fragments of C1q. Furthermore, incubation of ER4G-GMC with anti-mouse κ-chain antibodies also increased ER4G-mediated GMC-apoptosis. These results indicate for the first time that C1q enhances antibody-mediated apoptosis of rat GMC *in vitro*, presumably by its binding to ER4G and probably by additional cross-linking of Thy-1 on the surface of GMC.

Keywords apoptosis C1q complement Thy-1 rat glomerular mesangial cells

INTRODUCTION

The glomerular mesangial cell (GMC), a specialized pericyte originating from smooth muscle cells, plays a central role in maintaining the function of the glomeruli [1]. However, under certain conditions, activated GMC may produce inflammatory mediators such as platelet-derived growth factor (PDGF) [2–4], monocyte chemoattractant protein-I (MCP-I) [5,6], IL-1 [7], IL-6 [8,9], IL-8 [10], tumour necrosis factor-alpha (TNF-α) [11] and complement components [12,13]. Functionally altered GMC are thought to be involved in the pathogenesis of proliferative glomerulonephritis. For instance, some of the factors mentioned above are known to induce proliferation of GMC [3,4,8,9], a histological characteristic of proliferative glomerulonephritis. In contrast, recent studies also suggest that mesangial cell death by apoptosis (GMC-apoptosis), a different mode of cell death from necrosis, might also contribute to the course of glomerulonephritis in humans [15–18] and in animal models [18–21]. In an animal model of proliferative glomerulonephritis, the so called Thy-1 nephritis, the presence of GMC-apoptosis has been detected in the early phase by electron microscopy [19,21] and quantitatively

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analysed in the resolution phase of the disease by *in situ* end-labelling [20,21]. In *in vitro* studies, we and others previously reported that anti-Thy-1 MoAbs (ER4G and OX7) alone are able to induce apoptosis of rat GMC [22,23]. Further, we recently demonstrated that dimeric IgA anti-Thy-1 MoAb (ER4A) induces GMC-apoptosis more efficiently compared with the monomeric form, indicating that additional cross-linking of Thy-1 on the cell surface might be an important factor involved in the induction of GMC-apoptosis by anti-Thy-1 MoAb [24]. Since the classical pathway of complement plays an essential role in Thy-1 nephritis [25,26], and because apoptosis has been observed in the early phase of nephritis [19,21], we investigated whether binding of C1q, a subunit of the first component of complement, affects the anti-Thy-1-mediated apoptosis of rat GMC *in vitro*.

MATERIALS AND METHODS

Materials

Oxalic acid (Merck, Darmstadt, Germany), 2,2′-amino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), type Ia collagenase, trypsin, bovine serum albumin (BSA), RPMI 1640 (Seromed, Biochrom K.G., Berlin, Germany), fetal calf serum (FCS; GIBCO BRL, Grand Island, NY), propidium iodide (PI; Sigma Chemical Co., St Louis, MO), penicillin and streptomycin, Hoechst 33258 (Boehringer Mannheim, Mannheim, Germany), HiLoad 26/60 Superdex 200 prep grade, HiTrap Protein A, HiTrap Protein G (Pharmacia LKB Biotechnology, Uppsala, Sweden), pepsin (Worthington Biochemical Corp. Co., Freehold, NY), horseradish peroxidase-conjugated streptavidin (HRP-streptavidin; Zymed Labs Inc., San Francisco, CA), goat anti-mouse κ-chain antibody (GAM/κ) (Caltag Laboratory, San Francisco, CA) and FITC-labelled recombinant human annexin V (Bender MedSystems, Vienna, Austria) were purchased as indicated.

Culture of mesangial cells

GMC were isolated from cortical renal tissue of Sprague-Dawley rats using the sequential sieving method and enzyme dissociation of glomeruli by type Ia collagenase, as reported previously [27,28]. Isolated GMC were identified by morphological and immunohistochemical criteria, as before [27,28]. GMC were maintained in culture in T75 flasks (Greiner, GmbH, Frickenhausen, Germany) with complete medium (RPMI 1640 containing 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). For the apoptosis experiments, a defined number of GMC from passages 5 to 10 was seeded into 48-well plates (Costar, Cambridge, MA), cultured for 2 days in complete medium and rendered quiescent for 24 h in medium (RPMI 1640 containing 0·5% heat-inactivated FCS). At the beginning of the experiments, approximately 1×10^5 cells/well were present in a final volume of 0·5 ml of medium.

Mouse MoAbs

Mouse IgG2a anti-Thy-1 MoAb (ER4G) [21,29], IgG1 anti-Thy-1 MoAb (ER14) [26,29] and IgG2a anti-human C3bi receptor MoAb (IB4) [22] were purified from ascitic fluid by precipitation with saturated ammonium sulfate to a final concentration of 50%, followed by gel filtration on Superdex 200 prep grade columns. $F(ab')_2$ fragments of ER4G ($F(ab')_2$-ER4G) were prepared by pepsin digestion and removal of Fc parts and intact IgG by protein A column chromatography. SDS–PAGE analysis under non-reducing conditions demonstrated that the $F(ab')_2$ preparations were devoid of intact IgG [22]. The antibodies were kept in aliquots at -80°C and frozen and thawed maximally twice before use.

Isolation of C1q and collagen like fragments of C1q

C1q was isolated as described before [30]. Normal human serum (2500 ml) was precipitated by treatment with PEG-6000 to a final concentration of 3% (w/v) for 1 h on ice. After centrifugation, the pellet containing C1q was dissolved in 150 ml Veronal-buffered saline, followed by adjustment of conductivity to 12 mS with ice-cold water and the addition of EDTA to a final concentration of 2 mM. The solution containing C1q was applied on a rabbit IgG-Sepharose column prepared by overnight incubation at 4°C of human IgG-Sepharose with an excess of rabbit IgG anti-human IgG and subsequent washing with PBS. After extensive washing with PBS containing 2 mM EDTA, C1q was eluted with 1 M NaCl. Fractions containing C1q, as determined by C1q haemolytic assay [31] and C1q ELISA using rabbit anti-human C1q antibodies [32,33], were pooled. For the removal of IgG, the C1q preparation was applied on a column of protein G. The fall-through fractions containing C1q were collected, concentrated and dialysed against PBS. The final preparation was stored in glass tubes at −80°C until use. The purified C1q was shown to be haemolytically active and free of other serum proteins [34].

Collagen-like fragments of C1q (C-C1q) were prepared by pepsin digestion as described [35]. Briefly, C1q dialysed against 0·1 M sodium acetate buffer pH 4·5 containing 0·5 M NaCl was incubated with pepsin (1:30 (w/w)) at 37°C for 4·5 h. After stopping the digestion reaction with 1 M Tris solution pH 10·0, residual C1q was removed by absorption with rabbit IgG-Sepharose. The final preparation of C-C1q was dialysed against water, freeze-dried, dissolved in PBS and stored in glass tubes at −80°C until use. The C-C1q preparation contained no functional C1q haemolytic activity [34]. SDS–PAGE analyses under reducing conditions demonstrated that C-C1q was devoid of other detectable serum protein contaminants [34].

Binding of C1q to antibodies

For the detection of binding of isolated C1q to anti-Thy-1 MoAb, 96-well plates (Titertek, Zwanenburg, The Netherlands) were coated with 100 μl/well of each antibody at a concentration of 10 μg/ml in coating buffer pH 9·6 composed of 0·1 M Na_2CO_3 and 0·1 M $NaHCO_3$, overnight at room temperature. After washing with PBS containing 0·05% (v/v) Tween-20 (PBS–T), increasing concentrations of C1q were added to the wells and incubated for 1 h at 37°C. Following three washes with PBS–T, bound C1q were detected by biotin-conjugated rabbit anti-human C1q antibodies [32,33], using HRP-streptavidin. After final washes, HRP substrate solution (ABTS) was added and the colouring reaction was stopped by addition of 2% oxalic acid to each well after 30 min. Optical densities (OD) were measured at 415 nm using a Titertek Multiscan plate reader (Flow Labs, Zwanenburg, The Netherlands).

Induction of apoptosis in cultured rat GMC

Apoptosis of rat GMC was induced *in vitro* as reported before [24]. Briefly, quiescent rat GMC in 48-well plates were exposed for 1 h at 37°C to medium alone, 10 μg/ml of ER4G, ER14, $F(ab')_2$-ER4G or IB4 in medium. In this study, these cells will be designated as M-GMC, ER4G-GMC, ER14-GMC, $F(ab')_2$-ER4G-GMC or IB4-GMC, respectively. After washing with medium, cells were further incubated with medium alone or with medium in the presence of indicated concentrations of C1q, C-C1q or 1 μg/ml of GAM/κ for

the required time period at 37°C in 5% CO_2 and assessed for apoptosis. The concentration of 10 μg/ml of antibodies for loading the GMC was chosen from a dose–response curve giving suboptimal apoptosis with anti-Thy-1 MoAb alone. M-GMC incubated with medium alone was used as control.

Assessment of apoptosis

Morphological studies. To assess the nuclear changes in GMC morphologically, cells in the wells were collected by trypsinization and centrifugation, washed with PBS containing 1% BSA. After washing, cytospin preparations were made of the cells following fixation for 10 min with 1% paraformaldehyde. Thereafter the cells were stained with Hoechst 33258 at room temperature for 3 min. Nuclear changes such as condensation and fragmentation as detected with Hoechst 33258 in triplicate were evaluated by counting under fluorescence microscopy at ×400 magnification. At least 300 cells from each sample were counted. Further, in part of the experiments, to assess the membrane integrity of the cells showing nuclear changes qualitatively, double-staining procedures using Hoechst 33258 and PI were performed according to the method reported earlier, with slight modifications [22,36,37]. In brief, cells were collected as described above, washed with PBS containing 1% BSA and incubated with 10 μg/ml PI for 15 min at 30°C. Following fixation with 1% paraformaldehyde for 10 min, cytospin preparations were made of the cells, stained with Hoechst 33258 and immediately evaluated under fluorescence microscopy. Photographs were taken with camera and automatic timer.

Quantification by FACS. Apoptosis was assessed quantitatively by FACS using single staining with FITC-annexin V (annexin V method) according to the method established earlier [22,24,36,37]. After incubation, cells were collected as described above, washed with annexin V buffer composed of PBS containing 1% BSA and 1·8 mM $CaCl_2$, and labelled with 2·5 μg/ml of FITC-annexin V in the buffer for 15 min. At least 5000 cells from each sample were analysed. In time course experiments, bicolour analyses using 2·5 μg/ml of FITC-annexin V and 10 μg/ml of PI (annexin V/PI method) were performed [37]. In bicolour analysis, positive staining of the plasma membrane with FITC-annexin V in the absence of concomitant staining of nuclei with PI (AnV^+/PI^-) indicates apoptosis at early stages. Double-positive staining with both FITC-annexin V and PI (AnV^+/PI^+) suggests apoptosis at later stages, since PI can only enter cells and bind to the nuclei when damage to the membrane has occurred. At each time point, cells were collected by trypsinization and centrifugation, washed with annexin V buffer and incubated with a mixture of 2·5 μg/ml of FITC-annexin V and 10 μg/ml of PI in annexin V buffer for 15 min at 30°C. Following fixation with 1% paraformaldehyde for 10 min, fluorescence of the cells was measured immediately by FACS. At least 5000 cells from each sample were analysed.

Statistical analysis

Results were expressed as the mean ± s.d. Statistical analyses were performed using one-way analysis of variance with simultaneous multiple comparisons between groups by the Fisher's PLSD method. $P < 0{\cdot}05$ was considered significant.

RESULTS

Binding of C1q to anti-Thy-1 monoclonal antibodies

ER4G and ER14 are IgG2a [21,29] and IgG1 [26,29] subclasses of anti-Thy-1 MoAbs, respectively. To assess the binding of C1q to ER4G, ER14 and $F(ab')_2$-ER4G, 10 μg/ml of these proteins were fixed to 96-well plates, washed and incubated with increasing concentrations of C1q. The binding of C1q was determined by ELISA using polyclonal rabbit anti-human C1q antibodies [32,33]. Binding curves (Fig. 1) showed that C1q bound efficiently to ER4G, and in a dose-dependent fashion. Binding of C1q to ER14 was much less efficient. $F(ab')_2$-ER4G did not reveal considerable binding to C1q.

Effects of C1q on anti-Thy-1-mediated apoptosis

To investigate the effects of C1q on GMC-apoptosis, we first performed dose–response experiments to determine the dose of ER4G giving suboptimal apoptosis of GMC. A dose of 10 μg/ml was chosen. Rat GMC in 48-well plates were exposed to medium alone (M-GMC) or medium containing 10 μg/ml of ER4G (ER4G-GMC). After exposure, the GMC were washed and incubated for 16 h with medium alone or with medium containing 3·2, 12·5, 50 or 200 μg/ml of C1q. Apoptosis was assessed by FACS using the annexin V method (Fig. 2). M-GMC incubated with medium alone exhibited 14·2 ± 2·2% annexin V-positive cells, and incubation with C1q did not change the percentage of annexin V-positive cells significantly. ER4G-GMC incubated with medium alone resulted in 24·8 ± 4·9% apoptosis. C1q enhanced apoptosis of ER4G-GMC in a dose-dependent fashion and resulted in up to 58·6 ± 8·2% apoptosis at an input of 200 μg/ml C1q. A dose of 100 μg/ml of C1q, a dose corresponding to approximately the physiological serum concentration of C1q [38], was chosen for further experiments.

To determine the kinetic effect of C1q on GMC-apoptosis, M-GMC or ER4G-GMC were incubated with medium alone or with medium containing 100 μg/ml of C1q for 4, 8, 16 or 24 h. Cells were collected before exposure and at each time point during incubation. Apoptosis was assessed by FACS using the annexin V/PI method. Time-dependent increases in AnV^+/PI^- cells were

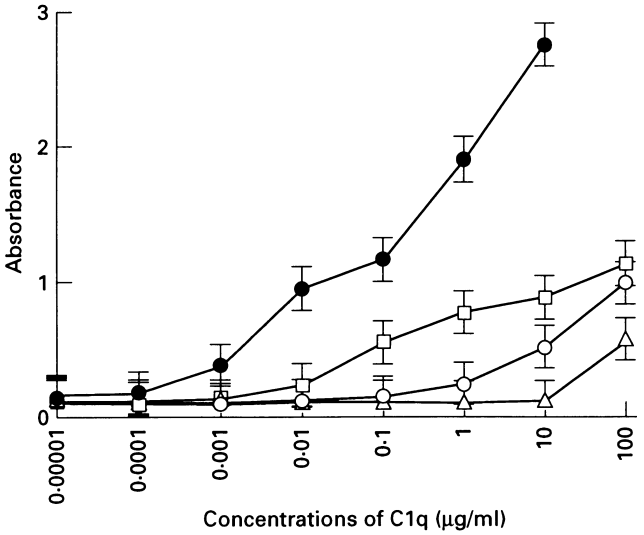

Fig. 1. Binding of C1q to anti-Thy-1 MoAb. ELISA plates were coated with 10 μg/ml of ER4G (IgG2a) (●), ER14 (IgG1) (□), $F(ab')_2$-ER4G (○) or bovine serum albumin (BSA) (Δ), washed and incubated with increasing concentrations of C1q. The binding of C1q was detected by biotin-conjugated rabbit anti-human C1q antibodies using horseradish peroxidase (HRP)–streptavidin. Data are expressed as the mean ± s.d. (n=3).

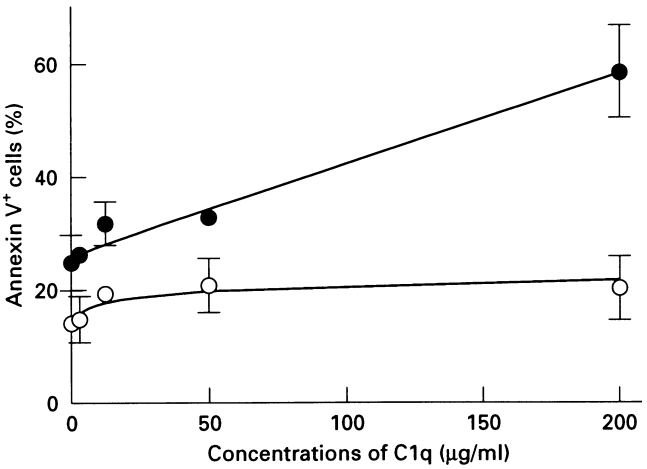

Fig. 2. Dose-dependent enhancing effect of C1q on apoptosis of ER4G-GMC. ER4G-GMC (●) and M-GMC (○) were incubated for 16 h with increasing concentrations of C1q. Apoptosis was assessed by FACS using the annexin V method as described. Data are expressed as the mean ± s.d. (n=3).

Table 1. Effect of C1q on nuclear changes in M-GMC and ER4G-GMC

	Apoptotic nuclear changes (%)*	
	Medium	C1q
M-GMC	6·7 ± 1·3	8·6 ± 2·5
ER4G-GMC	16·6 ± 1·6†	25·0 ± 3·3‡

After 16 h of incubation, cytospin preparations were made of the cells and stained with Hoechst 33258. Percentages of apoptotic nuclear changes such as nuclear condensation and fragmentation were determined under fluorescence microscopy.

* At least 300 cells were counted from each sample (n = 3).

† P < 0·01 relative to M-GMC incubated with medium alone.

‡ P < 0·01 relative to ER4G-GMC incubated with medium alone. There was no difference between M-GMC incubated with medium alone and C1q.

observed reaching plateau levels between 16 and 24 h. AnV^+/PI^+ cells, a measure of apoptosis at later stages, also increased with time. Apparent increases of AnV^+/PI^+ cells were detected only at later time points. The percentages of AnV^+/PI^+ cells were less than those of AnV^+/PI^- cells at each point (Fig. 3a). Incubation of M-GMC with medium alone or with C1q showed only slight changes in both AnV^+/PI^- cells and AnV^+/PI^+ cells. The highest percentages were around 10% both for AnV^+/PI^- cells and for AnV^+/PI^+ cells at 24 h points (Fig. 3b). On the basis of kinetic studies, incubation for 16 h was chosen to evaluate the effects of C1q on GMC-apoptosis.

Morphological assessment of apoptosis was performed in triplicate under fluorescent microscopy (Table 1). M-GMC (6·7 ± 1·3%) incubated for 16 h with medium alone showed nuclear changes compatible with apoptosis. Apoptotic nuclear changes in ER4G-GMC incubated with medium alone was 16·6 ± 1·6% (P < 0·01, relative to M-GMC incubated with medium alone). Incubation with C1q further significantly increased typical apoptotic nuclei to 25·0 ± 3·3% only in ER4G-GMC (P < 0·01 relative to ER4G-GMC incubated with medium alone), but not in M-GMC. To assess the membrane integrity of cells showing nuclear changes, ER4G-GMC were collected after incubation for 16 h with medium alone or with C1q and stained with Hoechst 33258 and PI simultaneously (Fig. 4). Although ER4G-GMC incubated with medium and with C1q revealed typical apoptotic nuclear changes with Hoechst 33258, a few of the cells showed apparently positive staining with PI. Further, PI-positive cells also exhibited typical apoptotic nuclear changes such as nuclear condensation and fragmentation.

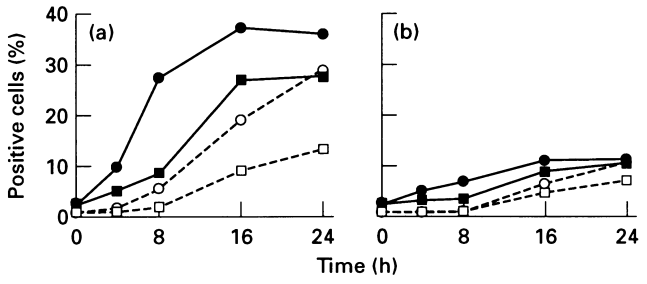

Fig. 3. Time-dependent enhancing effect of C1q on apoptosis of ER4G-GMC. ER4G-GMC (a) or M-GMC (b) were incubated for indicated time periods in the presence (circles) or absence (squares) of 100 μg/ml of C1q. Apoptosis was assessed by FACS using the annexin V/PI method as described in Materials and Methods. Closed and open symbols indicate FITC-annexin V-positive (AnV^+/PI^-) and double-positive FITC-annexin V and PI (AnV^+/PI^+), respectively. One representative experiment out of two independent experiments is shown.

After analyses of the dose- and time-dependent effects of C1q, experiments were performed in which M-GMC, ER4G-GMC, ER14-GMC, $F(ab')_2$-ER4G-GMC or IB4-GMC were incubated for 16 h with medium alone or with medium containing 100 μg/ml of C1q (Fig. 5a,b). Each experiment was performed in triplicate. M-GMC incubated with medium alone exhibited 9·4 ± 1·4% annexin V-positive cells. The presence of C1q had no significant effect on apoptosis of these M-GMC. ER14-GMC incubated with medium alone showed an increase of apoptosis to 19·7 ± 5·7% (P < 0·05). The presence of C1q did not significantly increase apoptosis of ER14-GMC. $F(ab')_2$-ER4G-GMC incubated with medium alone also showed a significant increase in apoptosis compared with M-GMC incubated with medium alone (P < 0·01). No additional effect of C1q on apoptosis of $F(ab')_2$-ER4G-GMC was observed. ER4G-GMC incubated without C1q resulted in 25·7 ± 5·7% apoptosis. Like ER14 and $F(ab')_2$-ER4G, this value was significantly higher than that of M-GMC incubated with medium alone (P < 0·01). Further, the presence of C1q significantly increased apoptosis of ER4G-GMC up to 39·4 ± 4·9% (P < 0·01 compared with ER4G-GMC incubated with medium alone) (Fig. 5a). IB4-GMC incubated with medium alone did not induce significant apoptosis, in agreement with a previous study [22]. C1q did not increase apoptosis of IB4-GMC (Fig. 5b)

Effect of collagen-like fragments of C1q and GAM/κ on apoptosis of ER4G-GMC

ER4G-GMC were incubated for 16 h with medium alone or with

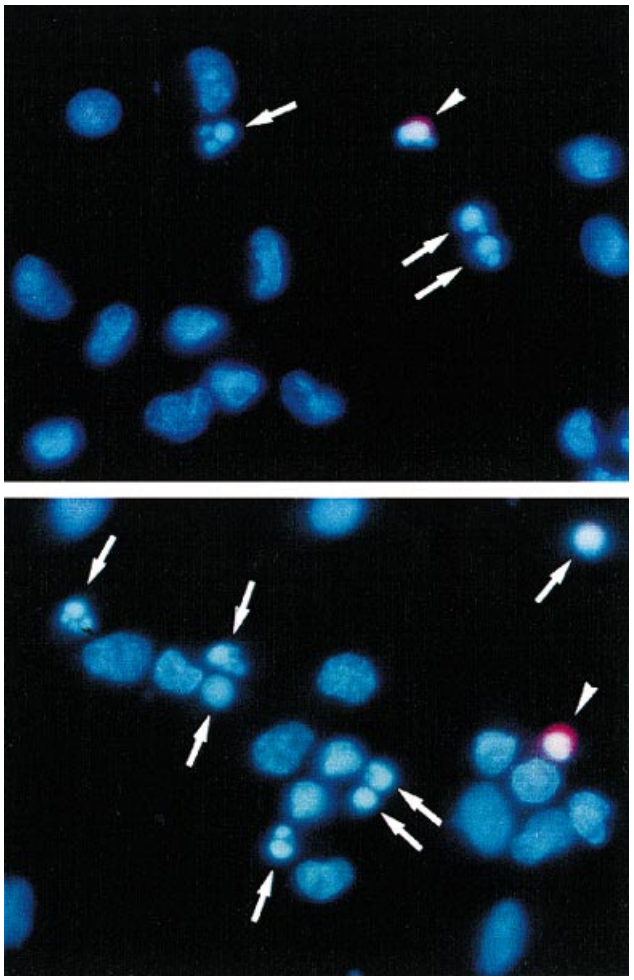

Fig. 4. Fluorescence microscopy of ER4G-GMC incubated with medium alone (top) and ER4G-GMC incubated with 100 μg/ml of C1q (bottom). Cells were incubated for 16 h under each condition and double-stained by Hoechst 33258 and propidium iodide (PI) as described in Materials and Methods. Arrows indicate apoptotic nuclei without positive staining of PI. Arrowheads indicate apoptotic nuclei with positive staining of PI. (Original mag., $\times$400.)

100 μg/ml of C1q or C-C1q. Apoptosis was assessed by FACS using the annexin V method (Fig. 6). All experiments were performed in triplicate. ER4G-GMC incubated with medium alone resulted in 21·9 $\pm$ 5·0% apoptosis. C1q again significantly enhanced ER4G-GMC apoptosis up to 32·9 $\pm$ 3·2% ($P < 0{\cdot}05$, compared with ER4G-GMC incubated with medium alone or with medium containing C-C1q). However, C-C1q did not significantly increase apoptosis of ER4G-GMC ($P = 0{\cdot}8$, compared with ER4G-GMC incubated with medium alone).

The results described above suggest that additional cross-linking of Thy-1 on cell surface augments the effect of ER4G on GMC-apoptosis. To investigate further whether this enhancing effect is observed generally by cross-linking, GAM/κ was used as the other linker. M-GMC and IB4-GMC or ER4G-GMC were incubated with or without GAM/κ in triplicates. After 16 h of incubation, apoptosis was determined by FACS using the annexin V method (Fig. 7a,b). ER4G-GMC incubated with medium alone showed 22·0 $\pm$ 3·2% apoptosis ($P < 0{\cdot}01$, relative to M-GMC incubated with medium alone). Again, ER4G-GMC incubated

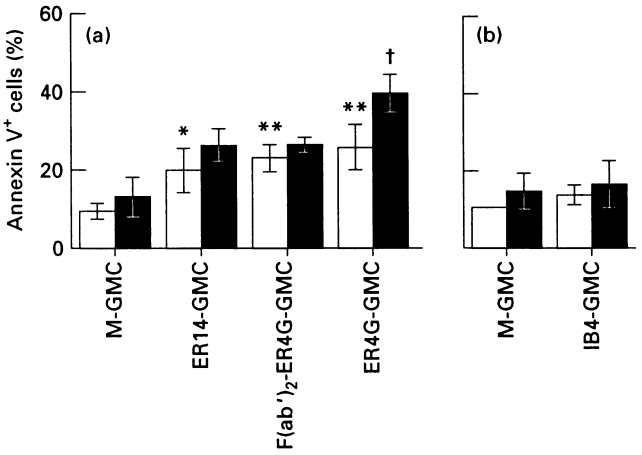

Fig. 5. Quantitative analyses of the effect of C1q on GMC-apoptosis by the annexin V method. M-GMC, ER14-GMC (a), F(ab′)$_2$-ER4G-GMC (a), ER4G-GMC (a) or IB4-GMC (b) were incubated for 16 h with medium alone (□) or with 100 μg/ml of C1q (■). Apoptosis was assessed by FACS as described in Materials and Methods. Data are expressed as the mean $\pm$ s.d. (n=3). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$. P values were calculated relative to M-GMC incubated with medium alone. †$P < 0{\cdot}01$. P value was calculated relative to ER4G-GMC incubated with medium alone.

with GAM/κ resulted in increase of apoptosis up to 43·1 $\pm$ 1·7% ($P < 0{\cdot}01$, relative to ER4G-GMC incubated with medium alone). However, GAM/κ did not have significant effects on either M-GMC or IB4-GMC.

DISCUSSION

We used human C1q to investigate the effect of C1q on GMC-apoptosis mediated by mouse anti-Thy-1 MoAb. Certain subclasses of mouse IgG are known to activate human complement via the classical pathway [39], suggesting that human C1q binds efficiently to these murine IgGs. Indeed, human C1q reacted

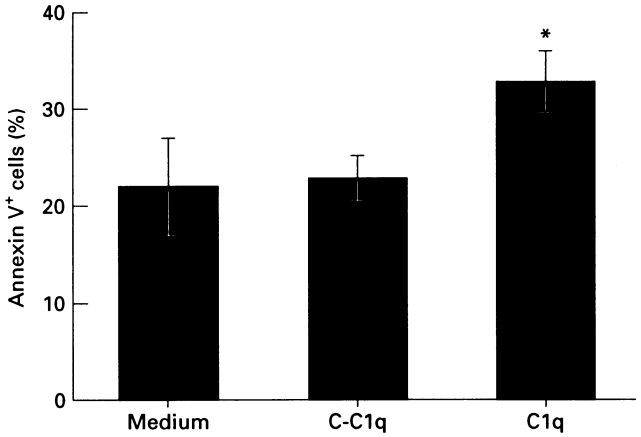

Fig. 6. Effect of collagen-like fragments of C1q (C-C1q) on apoptosis of ER4G-GMC. ER4G-GMC were incubated for 16 h with medium alone, with 100 μg/ml of C-C1q or C1q. Apoptosis was analysed by FACS using the annexin V method as described. Data are expressed as the mean $\pm$ s.d. (n=3). *$P < 0{\cdot}05$. P value was calculated relative to ER4G-GMC incubated with medium alone and with C-C1q.

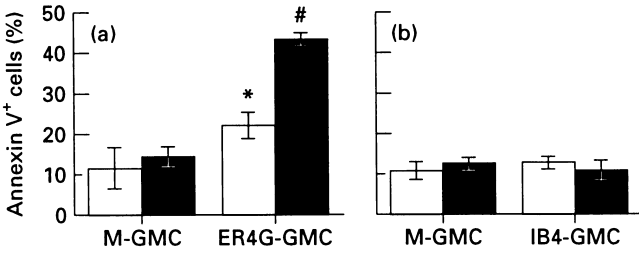

Fig. 7. Effect of goat anti-mouse κ-chain antibody (GAM/κ) on apoptosis of ER4G-GMC. M-GMC, ER4G-GMC (a) or IB4-GMC (b) were incubated for 16 h with medium alone (□) or with 1 μg/ml of GAM/κ in medium (■). Apoptosis was analysed by FACS using the annexin V method as described. Data are expressed as the mean ± s.d. (n=3). $*P < 0{\cdot}01$. P value was calculated relative to M-GMC incubated with medium alone. $\#P < 0{\cdot}01$. P value was calculated relative to ER4G-GMC incubated with medium alone.

efficiently with ER4G, a mouse IgG2a, as detected by ELISA. In contrast, ER14, a mouse IgG1, showed limited interaction with human C1q. $F(ab')_2$-ER4G did not reveal considerable binding to human C1q. These *in vitro* results are in agreement with previous *in vivo* studies in rats showing that the deposition of rat complement occurs upon injection of ER4G in the glomeruli [21], while ER14 does not induce detectable co-deposition of rat complement [26]. The reason for these differences in binding of C1q to ER4G, ER14 and $F(ab')_2$-ER4G is that the binding site of C1q is located in the Fc portion of immunoglobulins and that the binding of C1q to antibodies is dependent on the structure of the heavy chain of IgGs [40].

We and others have previously shown that apoptosis of rat GMC is specifically induced by anti-Thy-1 MoAbs (ER4G and OX7), not by OX18, an antibody against MHC-1, which also binds to cultured rat GMC [22,23]. The results of the present study demonstrate that C1q is able to enhance the ER4G-mediated apoptosis of rat GMC in a dose- and time-dependent manner, while no effect of C1q is seen with IgG2a isotype control (IB4), $F(ab')_2$-ER4G and ER14. Therefore, we postulate that C1q enhances ER4G-mediated GMC-apoptosis, presumably by more efficient cross-linking of Thy-1 on the surface of GMC. This assumption is in agreement with the experiments, in which intact C1q was necessary for enhancement of ER4G-mediated apoptosis of rat GMC: GAM/κ increased GMC-apoptosis of ER4G-GMC and not of IB4-GMC. Furthermore, the results, showing that C-C1q does not alter ER4G-mediated apoptosis of rat GMC, exclude a possible direct effect of the collagen part of C1q on apoptosis of rat GMC via occupation of C1q-receptors, which are known to be expressed both on human fetal [41] and rat GMC [42]. This conclusion is supported by our recent *in vitro* studies demonstrating that dimeric forms of IgA anti-Thy-1 MoAb (ER4A) induce apoptosis of rat GMC more efficiently than the monomeric form [24].

In the present study, apoptosis was quantified by FACS using the annexin V method. The annexin V method has been shown to detect early apoptosis preceding nuclear changes as measured by morphology, regardless of the cell type and initial stimulus of apoptosis [37]. Further, in our previous papers [22,24] and studies from others [36,43], results obtained by the annexin V method correlated well with DNA fragmentation by the agarose gel electrophoresis and the terminal deoxynucleotidyl transferase methods. One of the critical features of apoptosis is that apoptotic cells retain their plasma membrane integrity until later stages of apoptosis [44,45]. Therefore, in time course experiments, bicolour analyses using the annexin V/PI method were performed to distinguish the effect of C1q on apoptosis from primary necrosis. Since primary necrosis, as a result of, for example, the formation of complement C5b-9 complexes, first induces injury to plasma membranes [45] and, in contrast, plasma membrane damage of apoptotic cells generally occurs only at final stage of the process [36,37,44], we believe that this bicolour analysis is an accurate and quantitative method for this purpose. Indeed, at early time points we observed only AnV^+/PI^- cells. In contrast, AnV^+/PI^+ cells were detected at later time points only. Further, as reported in apoptosis of other cell types [36,37], there was an approximately 8-h difference in positivity of AnV^+/PI^- cells and AnV^+/PI^+ cells. These findings in the time course experiments, together with the results of morphological nuclear changes under fluorescence microscopy, indicate that C1q enhances ER4G-mediated GMC-apoptosis and that it does not increase concomitant primary necrosis.

In CS-21, a malignant lymphoma cell line, cross-linking of Thy-1 is reported to increase apoptosis [46]. Further cross-linking of other cell death molecules such as in the Fas–Fas ligand system [47] or TNF-mediated cell death [48] also results in enhancement of apoptosis. However, at present the intracellular mechanisms involved in anti-Thy-1-mediated apoptosis are unknown. Alterations of Bcl-2 protein expression in glomeruli are observed during mesangial proliferation and reduction of mesangial cellularity in several forms of human glomerular nephritis [16,17], suggesting involvement of Bcl-2 proteins in apoptosis of intraglomerular cells, including mesangial cells. However, it has been shown that KT16 and G7, both IgG2c anti-Thy-1 MoAbs, induce apoptosis of mouse thymocytes through bcl-2-resistant mechanisms [49]. Other anti-Thy-1 MoAbs, such as MCS-34, increase bcl-2 protein expression during induction of apoptosis in CS-21 cells [46]. Further, MCS-34 did not change the level of other bcl-2 protein family members such as bcl-X_L and bax [46], indicating that the bcl-2 protein family may not be involved in anti-Thy-1-mediated apoptosis. Other possible mechanisms may involve an increase of $[Ca^{2+}]_i$, which is involved in augmentation of endonuclease activity [50]. MCS-34 further cross-linked by secondary antibodies showed a significant increase of $[Ca^{2+}]_i$ during induction of apoptosis [46]. Recent studies in rat GMC using OX-7, known to induce apoptosis of rat GMC, and MoAb 1-22-3, an IgG3 anti-Thy-1 MoAb, demonstrate that both of the antibodies alone increase $[Ca^{2+}]_i$ [51]. However, since an increase of $[Ca^{2+}]_i$ has been established as an integral step in the signalling cascade for cell proliferation [52], change of $[Ca^{2+}]_i$ alone can not fully explain the intracellular mechanisms of anti-Thy-1-mediated apoptosis.

The present study indicates for the first time to our knowledge that C1q, a subunit of the first complement component of the classical pathway, is able to enhance anti-Thy-1-mediated GMC-apoptosis *in vitro*, presumably by additional cross-linking of Thy-1 on the GMC surface. This may result in a more efficient induction of GMC-apoptosis by complement-fixing anti-Thy-1 antibodies during complement activation via the classical pathway. Whether complement activation at stages later than C1q also has an additional effect on apoptosis requires further investigation.

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