The relationship between collagen and C1q biosynthesis in cultured human fibroblasts

Kenneth A. FLEMING and James O'D. McGEE

Nuffield Department of Pathology, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, U.K.

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The relationship between collagen and C1q biosynthesis has been investigated in cultured human fibroblasts. This was done by measuring the effects of variation in cell density, inhibition of prolyl hydroxylase and complexing of C1q on synthesis and/or secretion of both proteins. It was found that synthesis and secretion of both proteins were not co-ordinate, and that therefore regulation of expression of both proteins is probably not linked. However complexing of C1q did result in marked stimulation in collagen synthesis, suggesting that the fibrosis which follows inflammation may result from binding of C1q to the immune complexes formed in the inflammatory process.

Complement activation can occur via two pathways, the classical and the alternate (Müller-Eberhard, 1975). That initiated via C1, the first component of complement, is defined as classical pathway activation, while that initiated via factors B and D is called alternate pathway activation. C1 of the classical pathway is a calcium-dependent macromolecular complex of three protein subcomponents, C1q, C1r and C1s, of which C1q is responsible for initiating activation of the complex (Porter & Reid, 1978). The C1q molecule resembles a bunch of six tulips, the heads of which are thought to be responsible for recognizing and binding to immunoglobulin (Reid & Porter, 1976), and the bunched stems of which are thought to provide the sites at which C1r and C1s interact (Reid *et al.*, 1977). While the head region has a globular protein configuration, the stem region, which forms some 40% of the molecule, has a triple helical structure very similar to that of the collagenous proteins (Reid & Porter, 1976). The marked molecular similarity between C1q and the collagen proteins implies a degree of homology of the structural genes of the proteins. As a result, it has been suggested that C1q belongs to the family of collagen genes, and perhaps arose during duplication of those genes (Solomon, 1980). Since there is evidence to suggest that the expression of genes of a multigene family is linked (Brown, 1981), it may therefore follow that expression of the C1q and collagen genes is linked. This latter possibility would be of considerable interest, especially since inflammation, during which complement components, including C1q, are consumed, is

almost invariably followed by fibrosis, of which collagen synthesis and deposition is a principal component (Gay & Miller, 1978). Indeed many of the deleterious effects of inflammation are not caused by inflammation *per se*, but by the resultant fibrosis (McGee & Fallon, 1978). Although there is no direct evidence to prove the concept that expression of the C1q and collagen structural genes is linked, it is known that both proteins can be synthesized by the same cells [fibroblasts (Al-Adnani & McGee, 1976; Reid & Solomon, 1977)], and that synthesis of both proteins by these cells increases in certain inflammatory conditions *in vivo* (Al-Adnani & McGee, 1976). While this may be entirely fortuitous, it does suggest the possibility that regulation of expression of the C1q and collagen genes is linked.

One way of investigating whether C1q and collagen gene expression is linked is by determining whether collagen and C1q biosynthesis are co-ordinate, i.e., if synthesis or secretion of one protein is increased or decreased, does the synthesis or secretion of the other protein increase or decrease in a direct or reciprocal manner? Accordingly, we have investigated whether collagen and C1q biosynthesis are co-ordinate or not *in vitro*, by manipulating the environment in ways known to alter synthesis or secretion of one protein and measuring the effect on the other.

Materials and methods

Materials

Human embryonic skeletal parts were obtained from vacuum terminations. All tissue culture re-

Abbreviation used: IgG, immunoglobulin G.

agents and disposable plastic culture flasks were from Gibco-Biocult Ltd., Paisley, Scotland, U.K. Collagenase type III from *Clostridium histolyticum* (Sigma) was further purified in this laboratory to remove non-specific proteinase activity, the residual enzyme having specificity only for molecules with collagen sequences (Peterkofsky & Diegelmann, 1971).

Radiolabelled amino acids (The Radiochemical Centre, Amersham) included a high specific activity ^3H -labelled amino acid mixture, containing equal quantities by activity of: L-[4,5- ^3H]leucine (specific radioactivity $150\text{ Ci}\cdot\text{mmol}^{-1}$), L-[2,4,6- ^3H]phenylalanine ($65\text{ Ci}\cdot\text{mmol}^{-1}$), L-[4,5- ^3H]lysine ($77\text{ Ci}\cdot\text{mmol}^{-1}$), L-[2,3,4,5- ^3H]proline ($177\text{ Ci}\cdot\text{mmol}^{-1}$) and L-[2,3,5,6- ^3H]tyrosine ($87\text{ Ci}\cdot\text{mmol}^{-1}$). The concentration of these radiolabelled amino acids was $1\text{ mCi}\cdot\text{ml}^{-1}$. All other chemicals were of highest purity available and were obtained from either BDH, Sigma or Hopkins and Williams.

Detection of haemolytic activity and C1q synthesis

The cell lines, medium, culture conditions and assays for detection of haemolytic activity and C1q synthesis were as described (Fleming *et al.*, 1982).

Briefly, fibroblasts were isolated from human embryos and grown as monolayers in Eagle's minimal essential medium, Earle's salts and 10% foetal calf serum. C1 haemolytic activity was assayed by using EAC4 cells (sheep red blood cells coated with antibody and the fourth component of complement) according to Reid *et al.* (1977), and expressed as either percentage of complete lysis or as concentration of effective C1 molecules (Rapp & Borsos, 1970). C1q synthesis was assayed by pulse radiolabelling cell cultures and incubating the cell sonicates and medium with Sepharose beads coated with either goat monospecific anti-C1q IgG or ovalbumin. C1q synthesis was taken as the difference in radioactivity bound to each set of beads and expressed as c.p.m. per mg of DNA of each culture.

Total protein and collagen synthesis

Total protein and collagen synthesis in the cell layer and medium were determined as radioactivity incorporated into trichloroacetic acid-insoluble material and collagenase-degradable protein respectively, as described by Peterkofsky & Diegelmann (1971). They were expressed as c.p.m. per mg of DNA of the culture.

DNA content

The DNA content of the cultures was measured in an aliquot of the cell layer by the method of Burton (1956), using calf thymus DNA (Sigma) as a standard.

Preparation of prolyl hydroxylase (EC 1.14.11.2) from human skin

Crude prolyl hydroxylase was prepared from human skin according to the method of Rhoads & Udenfriend (1968). The enzyme preparation used was that obtained after the $(\text{NH}_4)_2\text{SO}_4$ precipitation step.

Preparation of heat-aggregated IgG

Rabbit anti-ovalbumin IgG (1 ml of $1.8\text{ mg}\cdot\text{ml}^{-1}$ in 150 mM -phosphate buffer, pH 8.0) was heated at 63°C for 20 min. The solution was centrifuged at 12000 g for 1 min at room temperature, and the supernatant was removed and stored at 4°C until used.

Manipulations of culture conditions

Cell density. Sixteen 25 cm^2 replicate flasks were seeded at $2\times 10^4\text{ cells}\cdot\text{cm}^{-2}$. The medium was changed each day and a proportion of the flasks was radiolabelled, in serum containing medium, for 3 h on days 1, 2, 3, 4, 5, and 8. Harvesting of the cell layer and medium, assay of DNA content, total protein, collagen and C1q synthesis was carried out as described above.

cis-4-Hydroxyproline. Four 25 cm^2 replicate flasks were seeded at $2\times 10^4\text{ cells}\cdot\text{cm}^{-2}$ and grown for 3 days. The medium was changed to fresh serum-free medium containing either *cis*-4-hydroxyproline or *trans*-4-hydroxyproline to a final concentration of $50\mu\text{g}\cdot\text{ml}^{-1}$. After incubation for a further 30 min at 37°C , the cells were pulsed for 3 h with radiolabelled amino acids and the cell layer and medium were harvested and assayed for DNA content, total protein, collagen and C1q synthesis.

α,α' -Dipyridyl. Fibroblasts were seeded at $2\times 10^4\text{ cells}\cdot\text{cm}^{-2}$ into four 25 cm^2 flasks and grown for 3 days. After a medium change, the cells were incubated for 30 min at 37°C and $10\mu\text{l}$ of ethanol, with or without α,α' -dipyridyl to a final concentration of 0.3 mM , was added; the cells were then pulsed for 3 h with radiolabelled amino acids in serum containing medium. The cell layer and medium were harvested and assayed for DNA content, total protein, collagen and C1q synthesis.

Ascorbate. Four 25 cm^2 replicate cultures were seeded at $2\times 10^4\text{ cells}\cdot\text{cm}^{-2}$ and grown for 3 days with daily changes of medium, but without fresh ascorbate. Then fresh serum-containing medium, with or without ascorbate, $50\mu\text{g}\cdot\text{ml}^{-1}$, was added and the cells were incubated for a further 1 h at 37°C . After this a 3 h pulse with radiolabelled amino acids was carried out, and the cell layer and medium were harvested and assayed for DNA content, total protein, collagen and C1q synthesis.

Dextran sulphate and heat-aggregated IgG. Six 25 cm^2 replicate flasks were seeded at $2\times 10^4\text{ cells}\cdot\text{cm}^{-2}$ and grown for 6 days, the medium being

changed after 3 days. Fresh serum-free medium was added on the sixth day. Phosphate buffer (200 μ l, 150 mM, pH 8.2) alone, or containing either dextran sulphate, final concentration 100 μ g $\cdot$ ml⁻¹, or heat-aggregated IgG, final concentration 72 μ g $\cdot$ ml⁻¹, was added to the appropriate flasks. Cells were incubated for 3 h at 37°C, then pulsed for 3 h with radio-labelled amino acids. The cell layer and medium were harvested and assayed for DNA content, total protein, collagen and C1q synthesis.

Ascorbate and accumulation of C1 haemolytic activity. Replicate 25 cm² flasks were seeded at 2×10^4 cells $\cdot$ cm⁻². After 24 h the medium was replaced with fresh medium, with or without ascorbic acid, to a final concentration of 50 μ g $\cdot$ ml⁻¹. An aliquot of the medium was taken after incubation with cells for 20 min at 37°C, and then the cells were cultured for 3 days. Fresh ascorbic acid was added daily to the appropriate flask, again to a final concentration of 50 μ g $\cdot$ ml⁻¹. After 3 days, the C1 haemolytic activity of both media was assayed.

Prolyl hydroxylation of fibroblast medium C1

Ascorbic acid (200 μ l of 50 mM), ferrous ammonium sulphate (200 μ l of 10 mM), catalase [40 μ l of 2.5% (w/v)] and 2-oxoglutarate (200 μ l of 100 mM) were added to 2 ml of fibroblast medium from a culture grown for 3 days in the absence of ascorbate. Purified prolyl hydroxylase (50 μ l), or 50 μ l of enzyme buffer, was added to 1 ml of the above mixture and incubated at 30°C for 4 h, after which C1 haemolytic activity was determined.

Results

Growth of the fibroblasts used in all experiments showed an initial 2 day lag phase, followed by exponential growth over 3 days and virtual cessation as confluence was reached. Total protein synthesis in both the cell layer and medium was maximal at the beginning of exponential growth, decreasing over the rest of the growth curve (results not shown). The patterns of collagen and C1q synthesis and accumulation were quite different however. Collagen synthesis was maximal in the cell layer at mid-exponential phase, the amount at other stages being much lower (Fig. 1a). In the medium, collagen was maximal at early and mid-exponential growth (Fig. 1a). C1q synthesis was maximal in the cell layer at 24 h, and showed a steep, but continuous drop till confluence was reached, when some levelling off occurred (Fig. 1b). Accumulation of C1q in the medium showed little variation with phase of cell growth, although there was a slight increase in early and mid-exponential phase (Fig. 1b).

All subsequent data and those given above were derived from cell pools and not on individual flasks. There was little variation in C1q synthesis from cell line to cell line, if the cells were examined under identical conditions. The major sources of variation were: the stage of growth at which cells were radiolabelled; the presence or absence of foetal calf serum during radiolabelling; the incubation conditions of the C1q assay. Within an experiment comparison of collagen and C1q biosynthesis was made under identical conditions.

Fig. 1. Collagen (a) and C1q (b) synthesis as a function of time in culture

See the Materials and methods section for experimental protocol and for assays of collagen and C1q synthesis.

●, Cell layer; ○, medium.

The effect of incubation of fibroblasts with *cis*-4-hydroxyproline is shown in Table 1. Collagen increased in the cell layer (17%) and decreased in the medium (25%). In contrast, both total protein and C1q decreased in both cell layer and medium, although C1q decreased slightly more than total protein, (12.5% compared with 9%). This slight decrease in C1q synthesis compared with total protein synthesis was consistent in two experiments.

The effect of 0.3 mM- α,α' -dipyridyl on total protein, collagen and C1q synthesis is shown in Table 2. Collagen increased (45%) in the cell layer, and decreased in the medium (41%) while, again in two sets of experiments, C1q showed a slight decrease in synthesis compared with total protein synthesis.

Synthesis of C1q, collagen and total protein by ascorbate-deficient fibroblasts, in the presence and absence of ascorbate, is shown in Table 3. Total collagen synthesis was unaffected, but on addition of ascorbate, the amount of collagen in the cell layer decreased by 47%, while that in the medium increased by 200%. In contrast, on addition of ascorbate, C1q increased in the cell layer (22.5%) with a corresponding decrease in the medium (28.5%). These alterations in collagen and C1q distribution between cell layer and medium were in marked contrast to the minimal changes in total protein distribution.

The effect of ascorbate deficiency on accumulation of C1q haemolytic activity in medium was also examined. As shown in Table 4, growing

fibroblasts in the absence of ascorbate results in a drop in the amount of C1 haemolytic activity detected, from 16.1×10^8 to 12.5×10^8 effective molecules $\cdot \text{ml}^{-1}$, a fall of approx. 22%. To determine if this drop in C1 haemolytic activity was due to relative underhydroxylation of proline in C1q, medium from ascorbate-deficient fibroblasts was incubated with prolyl hydroxylase and the effect was measured (Table 5). Functional C1q increased from 38.6% lysis to 60.3% lysis following prolyl hydroxylation.

Since the above procedures were aimed at inhibiting prolyl hydroxylase, it was necessary to check that the immunoassay could detect underhydroxylated C1q. Fibroblasts were therefore cultured and pulsed in the presence of 0.3 mM- α,α' -dipyridyl to inhibit prolyl hydroxylation. Aliquots of cell layer from these cultures were then exposed to purified prolyl hydroxylase (as described in Table 5) with or without 2-oxoglutarate and the amount of C1q detected by the immunoassay was measured. There was no difference between the control and hydroxylated C1q (results not shown), indicating that the immunoassay detected underhydroxylated C1q as effectively as it did the hydroxylated protein (no formal hydroxyproline estimations of the hydroxylated C1q were performed). This result was in contrast to that obtained for haemolytic activity (see above).

Table 6 shows the effect of addition of dextran sulphate to fibroblast cultures. Total protein

Table 1. *Effect of cis-4-hydroxyproline on total protein, collagen and C1q synthesis*

This experiment was performed as described in the Materials and methods section except that the medium did not contain foetal calf serum.

Addition to culture	$10^{-3} \times {}^3\text{H}$ incorporation (c.p.m. per mg of DNA) into:								
	Total protein			Collagen			C1q		
	Cell layer	Medium	Total	Cell layer	Medium	Total	Cell layer	Medium	Total
<i>trans</i> -4-Hydroxyproline	24 895	10 613	35 508	3112	4735	7847	2856	530	3386
<i>cis</i> -4-Hydroxyproline	23 000	9285	32 285	3654	3528	7182	2513	448	2961

Table 2. *Effect of α,α' -dipyridyl on total protein, collagen and C1q synthesis*

This experiment was performed in the presence of foetal calf serum as described in the Materials and methods section.

Addition to culture	$10^{-3} \times {}^3\text{H}$ incorporation (c.p.m. per mg of DNA) into:								
	Total protein			Collagen			C1q		
	Cell layer	Medium	Total	Cell layer	Medium	Total	Cell layer	Medium	Total
Nil	5421	1679	7100	218	100	318	309	6	315
α,α' -Dipyridyl	5164	1795	6959	316	59	375	260	4	264

Table 3. *Effect of ascorbate on total protein, collagen and C1q synthesis*

This experiment was performed in the presence of foetal calf serum as described in the Materials and methods section.

Addition to culture	$10^{-3} \times {}^3\text{H}$ incorporation (c.p.m. per mg of DNA) into:								
	Total protein			Collagen			C1q		
	Cell layer	Medium	Total	Cell layer	Medium	Total	Cell layer	Medium	Total
Nil	8337	2178	10 515	1226	350	1576	71	14	85
Ascorbate	8295	2507	10 802	649	1037	1686	87	10	97

Table 4. *Effect of ascorbate on accumulation of C1 haemolytic activity in medium*

This experiment was performed as described in the Materials and methods section.

Addition to fibroblast culture	Time (days)	Fibroblast medium (μl)	C1 haemolytic activity (% lysis)
Nil	0	50	0
		25	—
		12.5	—
	3	50	72.7*
		25	39.4
		12.5	16.9
Ascorbate	0	50	2.3
		25	—
		12.5	—
	3	50	83.3†
		25	50.9
		12.5	23.6

* Equivalent to 12.5×10^8 effective molecules $\cdot \text{ml}^{-1}$.

† Equivalent to 16.1×10^8 effective molecules $\cdot \text{ml}^{-1}$.

Table 5. *Effect of prolyl hydroxylase on fibroblast medium C1 activity*

This prolyl hydroxylase preparation had no endogenous C1 or C1q haemolytic activity when tested in this assay. Indeed it produced slight inhibition (approx. 10%) of a C1 preparation. Therefore the increase in fibroblast medium C1 activity produced by incubation with prolyl hydroxylase probably represents an underestimate. Prolyl hydroxylase was prepared, and C1 activity measured, as described in the Materials and methods section. Since 63% lysis was not achieved in this experiment, these data cannot be converted to effective molecules $\cdot \text{ml}^{-1}$.

Addition to fibroblast medium	Volume of medium (ml)	C1 haemolytic activity (% lysis)
Nil	1.0	38.6
	0.5	26.1
	0.25	0
Prolyl hydroxylase	1.0	60.3
	0.5	31.0
	0.25	0

Table 6. *Effect of dextran sulphate and heat-aggregated IgG on total protein, collagen and C1q synthesis*

This experiment was performed as described in the Materials and methods section but the medium did not contain foetal calf serum.

Addition to culture	$10^{-3} \times {}^3\text{H}$ incorporation (c.p.m. per mg of DNA) into:								
	Total protein			Collagen			C1q		
	Cell layer	Medium	Total	Cell layer	Medium	Total	Cell layer	Medium	Total
Nil	7953	6427	14 380	674	258	932	568	234	802
Dextran sulphate	11 206	6181	17 387	1229	716	1945	787	251	1038
Heat-aggregated IgG	10 652	3844	14 426	1295	328	1623	660	155	815

increased in the cell layer by 41%, while that in the medium barely altered (4% decrease). C1q showed much the same except that it increased slightly in the medium (7%). In contrast, collagen increased markedly in both cell layer (82%) and medium (177%). The addition of heat-aggregated IgG produced similar results, except that the decrease in total protein and C1q in the medium was more marked. After taking the effect on total protein synthesis into account, C1q synthesis was unaffected by addition of both dextran sulphate and aggregated IgG, while collagen synthesis was increased by approx. 75%.

Discussion

As described at the beginning of this paper, the joint possession of a unique tertiary molecular configuration, namely a triple helix, suggests that the biosynthetic pathways of collagen and C1q may be related. This possibility has been investigated by manipulating the culture conditions in ways known to alter synthesis or secretion of one protein, and measuring the effect on the other.

Total synthesis of collagen and C1q in cell layer and medium was compared under three different sets of experimental conditions. Initially the effect of cell density on both proteins was examined, as it is known that, *in vitro*, the amount of collagen synthesized decreases strikingly with increasing cell density (Berg *et al.*, 1976). In the experiments reported here, collagen synthesis did indeed vary with phase of cell growth, but, contrary to earlier studies on established cell lines (Berg *et al.*, 1976; Peterkofsky, 1972), it was maximal in mid-exponential phase cells, not in early exponential phase cells. On the other hand, C1q synthesis had a completely different pattern, being maximal in lag-phase cells. This is in contrast to both collagen and total protein synthesis, suggesting that C1q has its own pattern of synthesis.

The synthetic relationship between C1q and collagen was also examined by complexing C1q in the cultures by addition of heat-aggregated IgG and the polyanion dextran sulphate, both of which bind to C1q (Reid & Porter, 1976; Raeppe *et al.*, 1976). The results showed that both substances caused marked stimulation of collagen synthesis, but that C1q synthesis was unaffected after taking total protein synthesis into account [the possibility that complexed C1q could not be detected was avoided by including 50 mM-NaCl in the assay (Gabriel & Agnello, 1977)].

Lastly, the effects of inhibition of prolyl hydroxylase on collagen and C1q synthesis were investigated by addition of *cis*-4-hydroxyproline and α,α' -dipyridyl. These showed that C1q synthesis was slightly reduced, while collagen synthesis was unaf-

ected. This was more marked with α,α' -dipyridyl, and although the data were not striking, they were consistent between several experiments. Thus the results of the above set of three different experiments show that C1q and collagen biosynthesis are not co-ordinate with respect to synthesis.

The results of the experiments inhibiting prolyl hydroxylase also suggested that C1q and collagen biosynthesis are not co-ordinate with respect to secretion; inhibition of this enzyme markedly inhibits secretion of collagen (Prockop *et al.*, 1976). Initially functional inhibition of prolyl hydroxylase was carried out by addition of the proline analogue *cis*-4-hydroxyproline to the cultures (Prockop *et al.*, 1976). This produced a marked inhibition of secretion of collagen (approx. 20%), but C1q secretion was unaffected. Since *cis*-4-hydroxyproline does not inhibit prolyl hydroxylase catalytic activity directly, the experiment was repeated using α,α' -dipyridyl, which does inhibit enzyme activity by removal of a cofactor, viz. Fe^{2+} (Cardinale & Udenfriend, 1974). Addition of α,α' -dipyridyl inhibited collagen secretion (approx. 40%), but again C1q secretion was not inhibited. Depletion of ascorbate (another cofactor) produced similar results.

These results suggest that, unlike collagen, secretion of C1q is not dependent on prolyl hydroxylase activity. Therefore it appears that the metabolic pathways of C1q and collagen are not only non-co-ordinate with respect to synthesis, but are also non-co-ordinate with respect to secretion. Although this latter conclusion is at variance with previous publications (Müller *et al.*, 1978a,b), different assay, cell and culture conditions were involved.

Despite this, it does appear that prolyl hydroxylase does have a role in C1q metabolism. Thus depletion of ascorbate (a cofactor for prolyl hydroxylase) decreased accumulation of C1 haemolytic activity in medium. Incubation of this medium with purified prolyl hydroxylase partially restored activity. Since C1q is the only protein involved in C1 haemolytic activity with a prolyl residue which can be hydroxylated, this suggests that haemolytic activity of C1q depends on hydroxyproline content and prolyl hydroxylase activity. Using epithelial and fibroblast cell lines, Morris & Paz (1980) have reported similar findings. This does not necessarily mean however, that the collagen and C1q prolyl hydroxylase enzymes are identical, since different enzymes can have the same cofactor requirements e.g. prolyl hydroxylase and lysyl hydroxylase (Prockop *et al.*, 1979).

The demonstration in this paper that collagen and C1q biosynthesis is not co-ordinate with respect to synthesis and secretion *in vitro*, is supported by the findings that serum C1 haemolytic activity and the hydroxyproline and hydroxylysine content of C1q

are not altered *in vivo* by two diseases affecting collagen prolyl and lysyl hydroxylation, i.e. scurvy (Bates *et al.*, 1978) and Ehlers-Danlos type VI (Hanauske-Abel & Rohm, 1980) respectively.

Finally, as described in the introduction to this paper, inflammation often results in fibrosis. Although the processes by which inflammation produces fibrosis are not fully understood, it appears that products of immune responses acting directly, or indirectly, on the fibroblasts stimulate collagen synthesis (Allison *et al.*, 1977). In view of this, the action of dextran sulphate and heat-aggregated IgG on collagen synthesis is both striking and interesting. Although there are several other possibilities, it is tempting to suppose that the mechanism by which both dextran sulphate and heat-aggregated IgG increased collagen synthesis was related to their common property of C1q binding. Since immune complexes (which bind C1q) are formed in most inflammatory diseases, it is possible that the resultant complexing of C1q induces collagen synthesis, thus providing at least one of the mechanisms by which inflammation produces fibrosis.

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