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Co-deposition of clusterin with the complement membrane attack complex in myocardial infarction

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SUMMARY

Clusterin is a multi-functional plasma glycoprotein that has been shown to inhibit formation of the complement membrane attack complex (MAC) by preventing the association of terminal complement complexes with target cell membranes. Recent studies have suggested that complement activation is involved in the development of tissue injury of myocardial infarction. In this study we observed that clusterin is selectively deposited in the infarcted areas of human myocardium. Clusterin deposits were observed in the heart tissue of 10 patients whose infarcted lesions were 8 hr to 14 days old, but not in patients who died from other causes. Clusterin co-localized with the MAC on the surface of damaged cardiomyocytes. In normal myocardium only endothelial lining of blood vessels occasionally stained positive for clusterin. The 80,000 MW clusterin was also detected by Western blot analysis in extracts of myocardial infarction lesions, but only faintly in extracts of normal heart. As clusterin has apparently failed in protecting myocardium against complement-mediated cell injury its main role might be to participate in the clearance of damaged and necrotic tissue together with the MAC.

INTRODUCTION

Clusterin (SP40,40; Apo J; NA1/NA2; complement lysis inhibitor) is an 80,000 MW multi-functional glycoprotein that was first isolated from ram rete testis fluid, where it was described as being a major constituent with amphiphilic properties and an isoelectric point of 3·6 (ref. 1: reviewed in ref. 2). Clusterin has a tendency to aggregate cells, including Sertoli cells and erythrocytes.[1] Molecular cloning of human clusterin[3,4] indicated that it was a novel glycoprotein composed of two non-identical subunits of ≈40,000 MW. Clusterin has been shown to associate with the terminal complement (C) complexes and inhibit C lysis by preventing incorporation of the complexes to target cell membranes.[3–6] Clusterin associates with apolipoprotein A-I in high-density lipoprotein (HDL) particles in plasma suggesting a possible role in the regulation of lipid transport.[7–10] Deposits of clusterin have been detected in association with glomerular membrane attack complex (MAC) formation in human kidney diseases[5,11] and in experimental Heymann nephritis.[12] An increased mRNA expression and deposits of clusterin have been reported in brain tissue of patients with Alzheimer's disease.[13,14] In general, it has been suggested that clusterin could have an important function in tissue repair and remodelling as a response to tissue injury or to developmental or hormonal stimuli.[2–5,10,15–17]

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Studies in animal models and analysis of human autopsy specimens have shown that the C system will become activated in the infarcted areas of myocardium during acute myocardial infarction (AMI).[18–26] The cardiomyocytes are exposed to C during the reperfusion stage following an ischaemic injury. It is possible that C becomes activated spontaneously in the infarcted areas, because of the altered metabolic conditions and/or release of proteolytic enzymes. We have observed that protectin (CD59), an important membrane inhibitor of MAC, is selectively lost from the infarcted myocardial lesions.[24] Other membrane regulators of C, DAF, MCP and CR1, are only weakly or not at all expressed in the human myocardium.[27] Lack of protective membrane regulators could thus make the cardiomyocytes sensitive to C attack. In previous studies it has been observed that soluble C regulators, C4b binding protein, vitronectin (S-protein) and possibly, C8 binding protein, are co-deposited with the MAC in the infarcted lesions.[19,20,23,27] As a multi-functional protein capable of interacting with hydrophobic molecules clusterin, too, might be involved in the C membrane attack against myocardium. In the present study we tested this hypothesis and show that clusterin indeed is deposited in the infarcted myocardium together with the MAC.

MATERIALS AND METHODS

Patients

Human myocardial specimens were obtained at autopsies from 15 patients. Ten of the patients had an AMI leading to death

between 8 hr and 14 days from the initiation of symptoms. The control heart specimens were obtained from five patients without anamnestic or histopathological evidence of myocardial infarction. Seven of the AMI and three of the control patients have already been described in our earlier report.[24] Autopsies were carried out in the AMI group 1–6 days and in the control group 2–5 days after the death of the patient. The diagnosis of acute myocardial infarction was verified in a histopathological examination of heart specimens stained with haematoxylin–eosin.

Immunohistochemical staining

Areas of myocardial tissue displaying a typical infarction with ischaemic discoloration were selected at gross examination. For indirect immunofluorescence microscopy (IFL) the tissue blocks ($1 \times 1 \times 0{\cdot}5$ cm) were quickly frozen to $-65°$ using isopentane cooled with liquid nitrogen. Cryostat sections (4 μm) were fixed with cold acetone ($-20°$) for 10 min. The sections were incubated for 30 min at $+22°$ with mouse monoclonal antibodies (mAb) against clusterin and C5b–9 'neoantigens' (Quidel Corp., San Diego, CA). In addition, the sections were stained with rabbit antibodies against C1q, C3d, C4, and C9 (Behringwerke AG, Marburg, Germany) as described elsewhere.[27] After washing three times with phosphate-buffered saline, pH 7·4 (PBS), the sections were treated with fluorescein isothiocyanate (FITC)-conjugated antibodies against mouse or rabbit immunoglobulins (Dakopatts, Glostrup, Denmark). Control stainings were performed by omitting the primary antibody, by using non-immune sera or rabbit antibodies against haptoglobin, transferrin, IgA and IgG (Behringwerke AG). The IFL slides were mounted with Mowiol[28] and examined on a Zeiss Standard microscope equipped with a filter specific for FITC fluorescence.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Myocardial tissue samples (10–20 g) from infarcted lesions of two hearts with AMI and from two control hearts were rinsed thoroughly with ice-cold PBS, homogenized using an Ultra-Turrax homogenizer and solubilized with 60 mM octylglucoside (Sigma Chemical Co., St Louis, MO) in PBS for 1 hr at 0°. Insolubilized material was sedimented and the detergent extracts were diluted 1/10 in non-reducing SDS–PAGE buffer. Normal human serum and human seminal plasma diluted 1/100 in SDS–PAGE buffer were used as controls. SDS–PAGE under non-reducing conditions was performed according to the method of Laemmli[29] using 8% gels and a mini gel system (Bio-Rad Laboratories, Richmond, CA). After electrophoresis the proteins were transferred to nitrocellulose. Molecular weight standards were visualized by Ponceau S staining (Sigma). To prevent non-specific binding the nitrocellulose membrane was incubated with 3% bovine serum albumin (BSA) in PBS for 1 hr at 20° before incubation (30 min, 20°) with the anti-clusterin mAb diluted 1/100 in 3% BSA/PBS. The bound antibody was detected by alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibody (Orion-Diagnostica, Espoo, Finland) diluted 1/20 in 3% BSA/PBS. Nitroblue tetrazolium (NBT) (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (BCIP; Boehringer Mannheim, Mannheim, Germany) were used as substrates for alkaline phosphatase according to manufacturer's instructions.

RESULTS

Deposition of clusterin and complement components in normal and infarcted myocardium

Indirect immunofluorescence analysis of the distribution of clusterin and MAC was performed in 10 cases with and in five cases without acute myocardial infarction. In the control heart specimens no distinct myocardial deposits of clusterin were detected, but sometimes the coronary arteries and other blood vessels stained positive (mainly in the basement membranes). In the control hearts no C deposits in cardiomyocytes were observed, but basement membranes of blood vessels appeared occasionally positive for C3d and C9 irrespective of the time interval between death and autopsy (range 2–5 days) (Fig. 1f). In the infarcted hearts clusterin was observed in nearly all infarcted lesions in association with C9 (Fig. 1). The deposits stained positive also for the C5b–9 neoantigens, which are formed only after full assembly of the MAC on the target cell membranes (Fig. 2). In accordance with earlier studies components of the classical pathway of C (C1q, C3d and C4) were detected in the infarction lesions (Fig. 1).

The staining intensity of clusterin was strong in all infarction lesions aged from 8 hr to 14 days. In the early lesions (< 3 days) clusterin deposition co-localized with MAC mainly on the outer sarcolemmal surface of myocardial fibres (Fig. 3), while in the older lesions discrimination between intracellular and sarcolemmal deposits of C and clusterin was impossible because of cell necrosis (Fig. 1). In the older infarction lesions clusterin was sometimes only present in border areas between the outer part of the histologically identified myocardial lesion and normal adjacent myocardium. Also in these areas MAC co-localized strictly with clusterin. In the scars of old infarction lesions no deposits of C or clusterin were seen. Control stainings using anti-transferrin (Fig. 2), anti-haptoglobin, anti-IgG and anti-IgA antibodies, did not show selective staining of the infarcted myocardial lesions.

Western blotting analysis of clusterin in human myocardium

To examine whether the deposits of clusterin observed in the immunofluorescence analysis represented the intact clusterin molecule, infarcted areas strongly positive for clusterin in IFL were selected for Western blot analysis using the mouse anti-clusterin mAb.

Western blot of extracts from infarcted myocardial areas of two hearts showed a band of 80,000 MW after SDS–PAGE under non-reducing conditions (Fig. 4, lanes 4, 5). Only a very faint band in similar position was seen in extracts of two normal hearts (Fig. 4, lane 1). The size of the heart clusterin band corresponded with that in normal human serum (Fig. 4, lane 2) and seminal plasma (Fig. 4, lane 3). Although the intensities of the clusterin bands in seminal plasma and serum were stronger than in the extract of AMI heart the latter was clearly detectable.

DISCUSSION

The present study demonstrates, using IFL and Western blot analysis, that clusterin becomes deposited in the infarcted regions of human myocardium. Clusterin co-localized with the C9 component of the MAC (Fig. 1) and with the C5b–9 neoantigens (Fig. 2). Generation of the C5b–9 neoantigens and

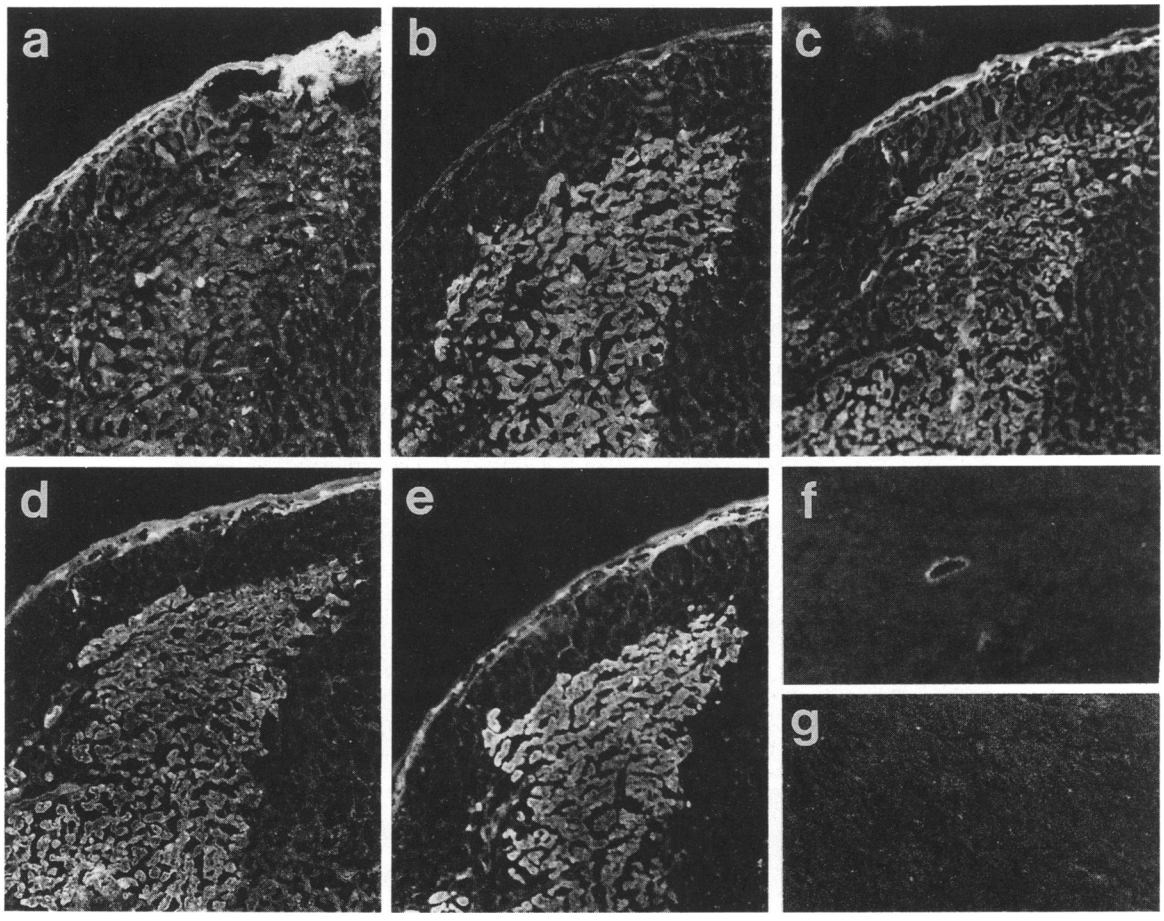

Figure 1. Immunofluorescence analysis of complement components and clusterin in a 5-day acute myocardial infarction lesion (a–e) and C9 in normal myocardium (f). Sequential cryostat sections were treated with rabbit antibodies against C1q (a), C4 (b), C3d (c), C9 (d, f) or with a mouse mAb against clusterin (e). Bound antibodies were detected with FITC-conjugated secondary antibodies. Infarcted myocardium shows co-deposition of complement components and clusterin. Normal myocardium did not stain for C9 except in a small artery (f). No staining of infarcted myocardium was observed when the primary antibody was omitted and the section stained with FITC-conjugated anti-mouse IgG (g). Magnification: × 75.

transmission electron microscopy observations have demonstrated that the MAC becomes fully assembled in the infarction lesions.[19,24,27] By specifically quantifying the soluble sC5b–9 and membrane C5b–9(m) complexes Hugo *et al.*[23] demonstrated that both forms of the terminal C complexes are present in the infarcted myocardium. Thus, by immunohistochemical analysis only, it is not possible to deduce whether clusterin is associated with the soluble or the membrane forms of the TCC or with both. The presence of fully assembled MAC in the lesions may indicate that MAC formation in the infarcted myocardium has proceeded despite the availability of clusterin.

The lack of reactivity of anti-transferrin and anti-immunoglobulin antibodies with the infarction lesions in the IFL analysis shows that not all serum proteins have become deposited in the infarcted lesions. Western blot analysis of samples from normal and infarcted myocardium further indicated that clusterin is selectively accumulated in the infarcted lesions. The molecular weight of clusterin in the lesions (80,000 MW) corresponded with that in serum and seminal plasma and with those earlier reported for various tissues.[2–5] The intensity of the clusterin band decreased in the order: seminal plasma > serum > AMI heart extract > control heart extract. Earlier investigations have shown that the concentration of clusterin is high (250–500 μg/ml) in seminal plasma where it is a major component with uncertain function.[3] The very faint band of clusterin in the non-infarcted heart may be because of serum clusterin trapped in capillaries. The results suggest that clusterin is weakly, if not at all, expressed in normal human heart tissue.

Clusterin in the infarcted lesions could originate from serum during reperfusion or alternatively, it could be synthesized by cardiomyocytes in response to ischaemia. The mRNA of clusterin (apo J) has been reported to be weakly expressed in the heart.[30] As clusterin was deposited in the earliest histologically detectable infarction lesions (approximately 8 hr old) it is likely that its accumulation, at least during the primary phase of myocardial infarction, is due to diffusion from plasma and not due to local synthesis in response to tissue injury. Whereas cells damaged during AMI are probably not able to synthesize clusterin, it is possible that viable cells surrounding the injured area could start expressing more clusterin mRNA and protein.

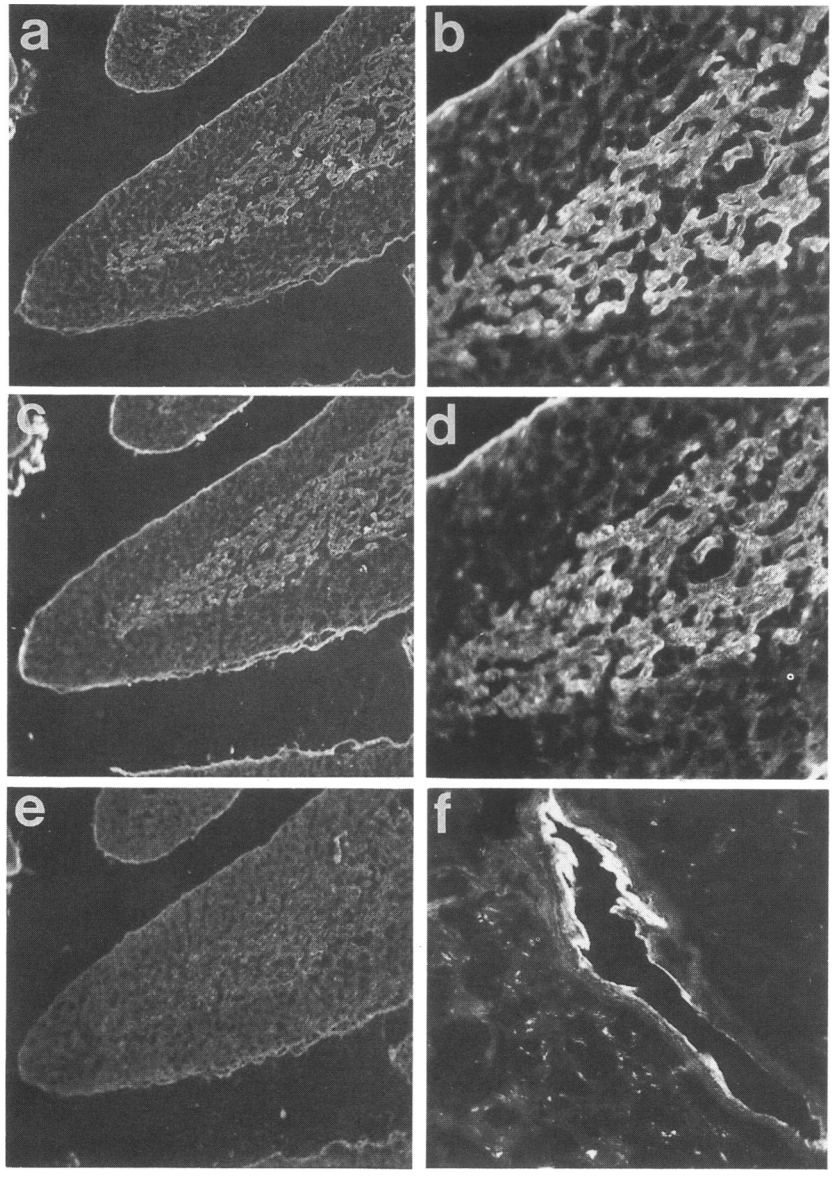

Figure 2. Immunofluorescence analysis of clusterin (a, b), C5b–9 neoantigens (c, d) and transferrin (e) in a 3-day infarction lesion in a papillary muscle. Clusterin and complement neoantigens were present in the infarcted lesion, but absent immediately beneath the endocardium. No deposits of clusterin were found in normal myocardium, except in a coronary blood vessel (f). Magnification: (a,c,e) ×52·5; (b, d, f) ×245.

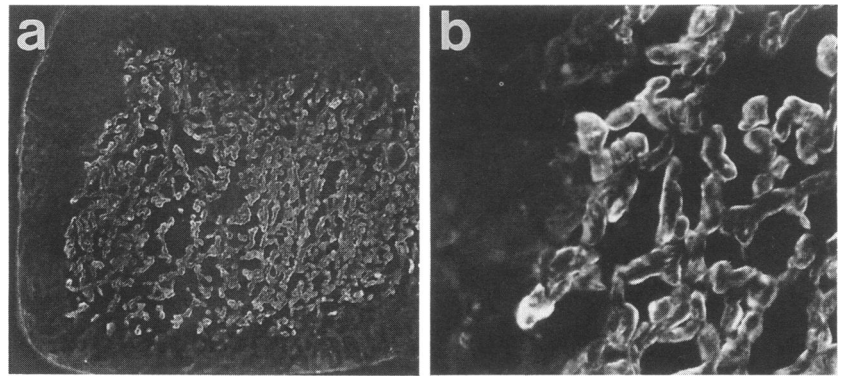

Figure 3. Immunofluorescence analysis of clusterin in a 2-day infarction lesion. Clusterin is deposited on sarcolemmal membranes on the outer surface of infarcted cardiomyocytes. Magnification: (a) ×52·5; (b) ×350.

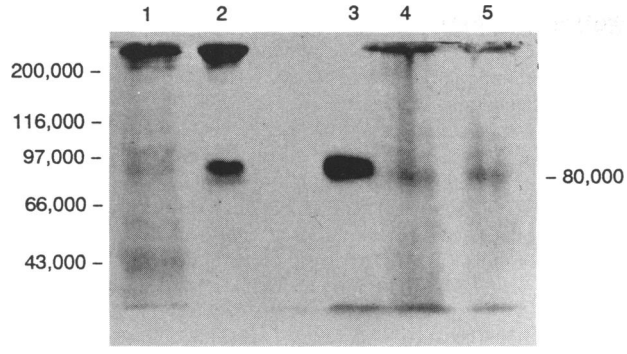

Figure 4. Western blot analysis of clusterin in non-infarcted myocardium (lane 1), normal human serum (lane 2), seminal plasma (lane 3) and infarcted myocardium (lanes 4, 5). Solubilized extracts of myocardial samples (diluted 1/10), serum (1/100) and seminal plasma (1/100) were subjected to 8% SDS–PAGE under non-reducing conditions, transferred to nitrocellulose and immunostained for clusterin using a mouse mAb. Clusterin appears as an 80,000 MW band in serum, seminal plasma and infarcted myocardium. The identity of the high molecular weight bands in lanes 1, 2, 4 and 5 is unknown, but they may represent non-specific reactivity of the mAb with material that has not entered the gel.

Increased expression of clusterin after tissue injury[31] could be needed for self-protection of the surrounding tissue or for the post-injury clearance and healing process.[27]

It is now evident that the resistance of human myocardium against MAC-mediated cell damage is regulated by several factors. Deposits of vitronectin (S-protein) co-localize with MAC as well as with clusterin in the infarcted lesions,[19,20,23,27] whereas the expression of protectin (CD59) has been shown to diminish.[24] Although it was initially proposed that the co-localization of vitronectin and clusterin with MAC deposition indicates the presence of a cytolytically inactive MAC, it has subsequently been shown that at least vitronectin can associate also with a cytolytically active MAC.[32,33] Currently it is uncertain whether any of the C regulators provide enough protective effect for cardiomyocytes during myocardial infarction, particularly as the main inhibitor of MAC, protectin, will be lost within 8 hr from the onset of AMI.[24] Another possibility is that the loss of protection against MAC attack and accumulation of the multi-functional proteins, clusterin and vitronectin, belong to a purposeful process whereby the injured tissue is eliminated and healing is initiated. When ischaemic cardiomyocytes lose their resistance against homologous C, the MAC will be formed on the sarcolemmal membranes and lead to their dissolution. The role of clusterin bound to the MAC might be to help in the clearance of MAC and the sarcolemmal membrane microparticles rather than to protect directly against C-mediated tissue injury. As an extension, it is possible that clusterin could have a general role in the recycling of material from damaged cells after various forms of cell injury.

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REFERENCES

1. Blaschuk O.W., Burdzy K. & Fritz I.B. (1983) Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. *J. biol. Chem.* **12,** 7714.
2. Jenne D.E. & Tschopp J. (1992) Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem. Sci.* **17,** 154.
3. Jenne D.E. & Tschopp J. (1989) Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc. natl. Acad. Sci. U.S.A.* **86,** 7123.
4. Kirszbaum L., Sharpe J.A., Murphy B., D'Apice A.J.F., Classon B., Hudson P. & Walker I.D. (1989) Molecular cloning and characterization of the novel human complement-associated protein, SP-40,40: a link between the complement and reproductive systems. *EMBO J.* **8,** 711.
5. Murphy B.F., Kirszbaum L., D'Apice A.J.F., Classon B., Hudson P. & Walker I.D. (1989) SP40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in immune deposits in glomerulonephritis. *J. clin. Invest.* **81,** 1858.
6. Choi N.H., Nakano Y., Tobe G., Mazda H. & Tomita M. (1990) Incorporation of SP-40,40 into the soluble membrane attack complex (SMAC, SC5b-9) of complement. *Int. Immunol.* **2,** 413.
7. de Silva H.V., Stuart W.D., Park Y.B., Mao S.J.T., Gil C.M., Wetterau J.R., Busch S.J. & Harmony J.A.K. (1990) Purification and characterization of apolipoprotein J. *J. biol. Chem.* **265,** 14292.
8. de Silva H.V., Stuart W.D., Duvic C.R., Wetterau J.R., Ray M.J., Ferguson D.G., Albers H.W., Smith W.R. & Harmony J.A.K. (1990) A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. *J. biol. Chem.* **265,** 13240.
9. Ehnholm C., Bozas S.E., Tenkanen H., Kirszbaum L., Metso J., Murphy B. & Walker I.D. (1991) The apolipoprotein A-I binding protein of placenta and the SP-40,40 protein of human blood are different proteins which both bind to apolipoprotein A-I. *Biochim. biophys. Acta,* **1086,** 255.
10. Jenne D.E., Lowin B., Peitsch M.C., Böttcher A., Schmitz G. & Tschopp J. (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. *J. biol. Chem.* **266,** 11030.
11. French L.E., Tschopp J. & Schifferli J.A. (1992) Clusterin in renal tissue: preferential localization with the terminal complement complex and immunoglobulin deposits in glomeruli. *Clin. exp. Immunol.* **88,** 389.
12. Eddy A.A. & Fritz I.B. (1991) Localization of clusterin in the epimembranous deposits of passive Heymann nephritis. *Kidney Int.* **39,** 247.
13. May P.C., Lampert-Etchell S.M., Johnson S.A., Poirier J., Masters J.N. & Finch C.E. (1990) Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer disease and in response to experimental lesions in rat. *Neuron,* **5,** 831.
14. McGeer P.L., Kawamata T. & Walker D.G. (1992) Distribution of clusterin in Alzheimer brain tissue. *Brain Res.* **579,** 337.
15. Buttyan R., Olsson C.A., Pintar J., Chang C., Bandyk M., Ng P.Y. & Sawczuk I.S. (1989) Induction of the TRPM-2 gene in cells undergoing programmed death. *Mol. cell. Biol.* **9,** 3473.
16. Rosenberg M.E. & Paller M.S. (1991) Differential gene expression in the recovery from ischemic renal injury. *Kidney Int.* **39,** 1156.
17. Cheng C.Y., Mathur P.P. & Grima J. (1988) Structural analysis of clusterin and its subunits in ram rete testis fluid. *Biochemistry,* **27,** 4079.
18. Pinckard R.N., O'Rourke R.A., Crawford M.H., Grover F.L., McManus L.M., Ghidoni J.J., Storrs S.B. & Olson M.S. (1980) Complement localization and mediation of ischemic injury in baboon myocardium. *J. clin. Invest.* **66,** 1050.

19. Schäfer H., Mathey D., Hugo F. & Bhakdi S. (1986) Deposition of the terminal C5b–9 complement complex in infarcted areas of human myocardium. *J. Immunol.* **137,** 1945.
20. Rus H.G., Niculescu F. & Vlaicu R. (1987) Presence of C5b–9 complement complex and S-protein in human myocardial areas with necrosis and sclerosis. *Immunol. Lett.* **16,** 15.
21. Crawford M.H., Grover F.L., Kolb W.P., McMahan C.A., O'Rourke R.A., McManus L.M. & Pinckard R.N. (1988) Complement and neutrophil activation in the pathogenesis of ischemic myocardial injury. *Circulation,* **78,** 1449.
22. Kagiyama A., Savage H.E., Michael L.H., Hanson G., Entman M.L. & Rossen R.D. (1989) Molecular basis of complement activation in ischemic myocardium: identification of specific molecules of mitochondrial origin that bind human C1q and fix complement. *Circ. Res.* **64,** 607.
23. Hugo P., Hamdoch T., Mathey D., Schäfer H. & Bhakdi S. (1990) Quantitative measurement of SC5b–9 and C5b–9(m) in infarcted areas of human myocardium. *Clin. exp. Immunol.* **81,** 132.
24. Väkevä A., Laurila P. & Meri S. (1992) Loss of expression of protectin (CD59) is associated with complement membrane attack complex deposition in myocardial infarction. *Lab. Invest.* **67,** 608.
25. Maroko P.R., Carpenter C.B., Chiariello M., Fishbein M.C., Randvany P., Kostman J.D. & Hale S.L. (1978) Reduction by cobra venom factor of myocardial necrosis after coronary artery occlusion. *J. clin. Invest.* **61,** 661.
26. Weisman H.F., Barton T., Leppo M.K., Marsh H.C. JR., Carson G.R., Concino M.F., Boyle M.P., Roux K.H., Weisfeld T.M.L. & Fearon D.T. (1990) Soluble human complement receptor type 1: *in vivo* inhibition of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science,* **249,** 146.
27. Väkevä A., Laurila P. & Meri S. (1993) Regulation of complement membrane attack complex formation in myocardial infarction. *Am. J. Path.* **143,** 65.
28. Heimer G.V. & Taylor C.E.D. (1974) Improved mountant for immunofluorescence preparations. *J. clin. Pathol.* **27,** 254.
29. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature,* **227,** 680.
30. de Silva H.V., Harmony J.A.K., Stuart W.D., Gil C.M. & Robbins J. (1990) Apolipoprotein J: structure and tissue distribution. *Biochemistry,* **29,** 5380.
31. Correa-Rotter R., Hostetter T.H., Nath K.A., Manivel J.C. & Rosenberg M.E. (1992) Interaction of complement and clusterin in renal injury. *J. Am. Soc. Nephrol.* **3,** 1172.
32. French L.E., Polla L.L., Tschopp J. & Schifferli J.A. (1992) Membrane attack complex (MAC) deposits in skin are not always accompanied by S-protein and clusterin. *J. invest. Dermatol.* **98,** 758.
33. Bhakdi S., Kaflein R., Halstensen T.S., Hugo F., Preissner K.T. & Mollnes T.E. (1988) Complement S-protein (vitronectin) is associated with cytolytic membrane bound C5b–9 complexes. *Clin. exp. Immunol.* **74,** 459.