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Biosynthesis and secretion of the third component of complement by human endothelial cells *in vitro*

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SUMMARY

The third component of complement (C3) is synthesized and released by cultured human capillary endothelial cells. After the incubation of cells in a methionine-free medium containing ^{35}S-methionine for 48 hr, culture supernatants were immunoprecipitated with anti-human C3 serum. SDS solubilization and 2-ME reduction of the immunoprecipitates, followed by separation with SDS–polyacrylamide gel electrophoresis and autoradiography, revealed two major bands comparable to those of the α and β chains of human C3. The content of C3 in the culture medium harvested at different time-intervals was determined by ELISA. The C3 secretion rate was about 250 ng/10^6 cells/5 days in the primary culture medium and 75 ng/10^6 cells/5 days after seven passages. The capillary endothelial cells described here have factor VIIIRAg specific for endothelial cells, and exhibit ring formation resembling capillary lumina, but they lack Weibel–Palade bodies.

INTRODUCTION

The genetics of complement have been extensively investigated in man. Three complement components, factor B, C2 and C4, have been reported to be closely linked to the HLA-B locus on chromosome 6 (Arnason *et al.*, 1977; Fu *et al.*, 1974; Teisberg *et al.*, 1977). In the case of C3, the electrophoretic variants or the inherited deficiencies have been reported not to be linked to HLA (Gedde-Dahl, Teisberg & Thorsby, 1974). Whitehead *et al.* (1982) confirmed the assignment of the human C3 genes to chromosome 19, and speculated that, after divergence of an ancestral gene to C3 and C4, the C4 gene was transposed into the ancestral major histocompatibility system, and that the C3 gene in man must have been translocated from chromosome 6 to chromosome 19. In addition, C3, C4 and C5 are synthesized as single-chain precursor proteins that undergo proteolytic cleavage to generate native multichain proteins (Brade, Hall & Colten, 1977; Hall & Colten, 1977; Ooi *et al.*, 1979). Colten, Ooi & Edelson (1979) and Whitehead *et al.* (1982) hypothesized that the marked similarities between C3, C4 and C5 indicate a common ancestral gene from which all three have arisen by tandem duplication.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HRP, horseradish peroxidase; MEM, minimum essential medium; 2-ME, 2-mercaptoethanol; PAGE, polyacrylamide gel electrophoresis; PAP, peroxidase–anti-peroxidase; PMSF, phenylmethylsulphonylfluoride; VIIIRAg, VIII-related antigen; SDS, sodium laurylsulphate; TAPM, tris(4-aminophenyl)methane.

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The major sites of synthesis for C3 are known to be mononuclear phagocytes (Einstein *et al.*, 1977) and liver cells (Alper *et al.*, 1969). It has also been reported that C3 is synthesized and secreted by primary cultured human fibroblasts (Whitehead *et al.*, 1982). The authors intend in this report to demonstrate the capability of human endothelial cells to biosynthesize and release C3 *in vitro*.

MATERIALS AND METHODS

Reagents

Goat anti-human C3 serum, goat PAP and goat HRP anti-rabbit IgG were purchased from Cappel Lab. Inc., Cochranville, PA. Rabbit anti-human C3 IgG (MBL Co., Nagoya, Japan), goat anti-human factor VIIIRAg antiserum (Nordic, Tilburg, The Netherlands), and purified human complement component C3 (Cordis Lab. Miami, U.S.A.), ABTS (MBL) and ^{35}S-methionine (Amersham International, Amersham, Bucks, U.K.) were used for the experiments.

Cell culture

Endothelial cells from human skin were cultured from part of a hypertrophic scar of a healthy 9-year-old male donor. Specimens were cut into small pieces, put into petri-dishes and covered with glass coverslips. Dulbecco's modified MEM supplemented with 10% FCS, penicillin at 100 units/ml and streptomycin at 100 μg/ml were fed and incubated at 37° in a humidified atmosphere of 5% CO_2 and maintained as usual. Culture supernatants for ELISA were collected, centrifuged and stored at $-70°$ until use.

Immunofluorescent staining of factor VIIIRAg
In order to identify the endothelial origin of the cells, factor VIIIRAg was examined by the indirect immunofluorescent antibody technique.

PAP staining of factor VIIIRAg
PAP staining for human factor VIIIRAg was performed as usual.

ELISA for quantification of C3
For quantification of C3, the wells in microtitre plates (Dynatech, Lab. Inc., Chantilly, U.S.A.) were coated with goat anti-human C3 serum for 2 hr at 37°. After washing, purified C3 or culture supernatants were incubated in the wells for 30 min at 37°. The plates were treated with rabbit anti-human C3 IgG, HRP-labelled goat anti-rabbit IgG globulin, and ABTS as a substrate for HRP. The enzyme activity was detected under an autoreader (Dynatech). In order to verify the specificity of the reaction in ELISA, a blocking test was carried out in the following manner. After binding of C3 to the wells, the goat antiserum used for coating the wells was poured into them and incubated for 30 min at 37°. The plates were washed thoroughly and treated as usual. Absorbance of the samples at 405 nm was compared, with and without a blocking test.

Inhibition of C3 synthesis by cycloheximide
Cultures were grown in plastic culture bottles for 5 days. The medium was replaced with a medium containing 5 μg/ml of cycloheximide. Supernatants were used for the quantification of released C3 with ELISA on the fifth day.

Radiolabelling of cell products
Cell cultures grown in 75-cm^2 flasks were radiolabelled with 2·5 ml of methionine-free Dulbecco's MEM containing 0·5 mCi (18·5 MBq) of ^{35}S-methionine but no FCS. After 48 hr, supernatants were centrifuged and stored at −70° until use.

Immunoprecipitation
Purified C3 as a carrier, 150 μl of ^{35}S-labelled culture supernatants and antiserum calculated to be equivalent to the amount of C3 were mixed. To the reaction mixture were also added KCl, EDTA and PMSF to final concentrations of 0·5 M, 0·01 M and 1 mM, respectively. The mixture was incubated overnight at 4°. The precipitates were centrifuged and boiled for 5 min in 10 μl of 0·025 M Tris/HCl buffer, pH 6·8, containing 2% SDS, 10% glycerol, 0·03% bromophenol blue, and 5% 2-ME for reduction of the samples.

SDS–PAGE and autoradiography
Precipitated materials were subjected to SDS–PAGE using the method of Laemmli (1970). For autoradiography, the stained gels were dried and exposed to XR-L X-ray film (Fuji, Tokyo, Japan) at −70° for 2 weeks.

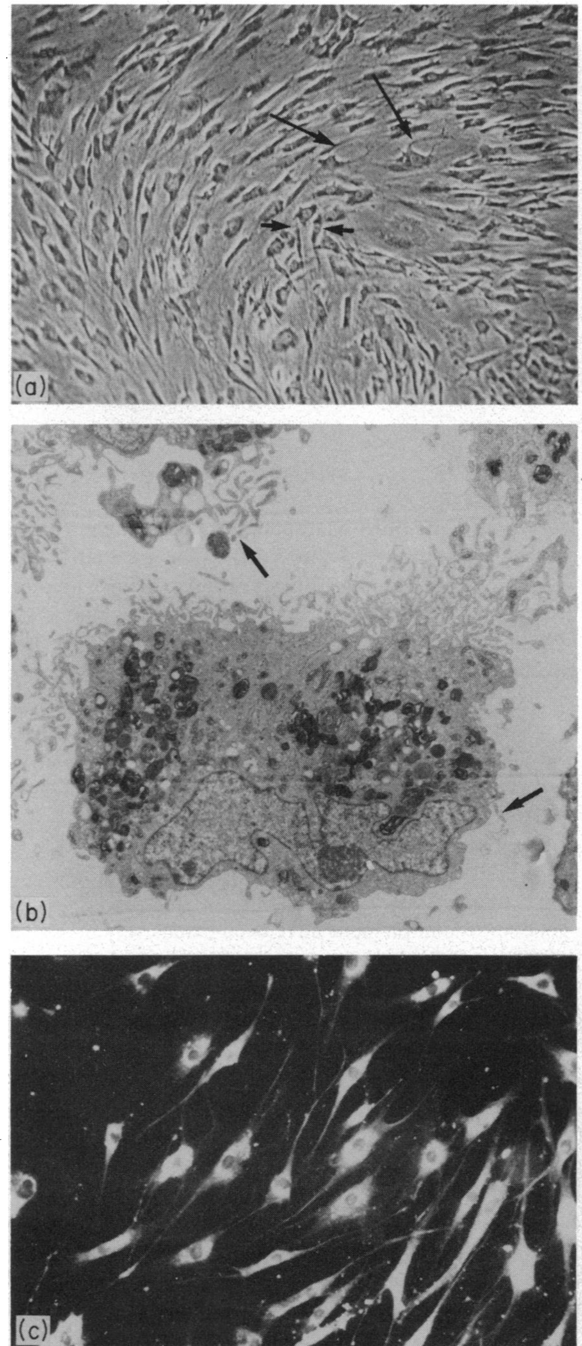

Figure 1. (a) Phase-contrast micrograph: ring formation and tube formation by the cells (magnification ×210). (b) Transmission electron micrograph: arrows indicate fenestra (magnification ×4200). (c) Immunofluorescent antibody staining for factor VIIIRAg (magnification ×280).

RESULTS

Identification of the endothelial origin of cells

Cells in culture were identified as endothelial in origin by the presence of factor VIIIRAg detected by the indirect immunofluorescent antibody technique and PAP staining (Fig. 1c). The cells seemed to be a homologous population because of the positive staining of almost 100% of the cells by the immunofluorescent antibody technique. In addition, they were also identified as endothelial cells by their characteristic cellular morphology. Formation of capillary segments or longitudinal tubes could be observed, as shown in Fig. 1a. Examination by electron microscope revealed them to be characterized by the

presence of typical fenestra, a characteristic of endothelial cells. Weibel–Palade bodies, however, could not be detected (Fig. 1b).

Release of the third component of complement *in vitro*

Supernatants from the primary culture of cells contained relatively large amounts of C3, 250 ng/10^6 cells/5 days, and 75 ng/10^6 cells/5 days after seven passages. Even after 13 passages of the cells, C3 was still detectable in culture medium with ELISA. After the blocking test to verify the immunological specificity of the reaction in ELISA, the optical density of the reaction decreased markedly. This indicates that the reaction in ELISA was immunologically specific, and that it reflected the amount of C3 bound to the wells. The control culture medium, which contained 10% FCS and which was not incubated with the cells, showed no human C3 with ELISA. The release of C3 from the cells decreased gradually after repeated passages of the cells due to their ageing.

Influence of cycloheximide on the synthesis and secretion of C3

Cycloheximide at a concentration of 5 μg/ml strongly inhibited the synthesis of C3 over a 5-day period (Fig. 2b). The amount of C3 released into medium from cycloheximide-treated cells was about 1·7% of that from untreated cells. The inhibition was reversible, and the cells could release C3 without cycloheximide again.

Immunoprecipitation and SDS–PAGE analysis of synthesized C3

Immunoprecipitated C3 was resolved as characteristic bands representing the C3α and β chains analysed by SDS–PAGE after SDS solubilization and 2-ME reduction. Autoradiography of the gel and staining with Coomasie Blue of the same sample subjected to SDS–PAGE in the same manner are shown in Fig. 3. The ^{35}S-labelled α and β bands had the same molecular weight as in the stained gel.

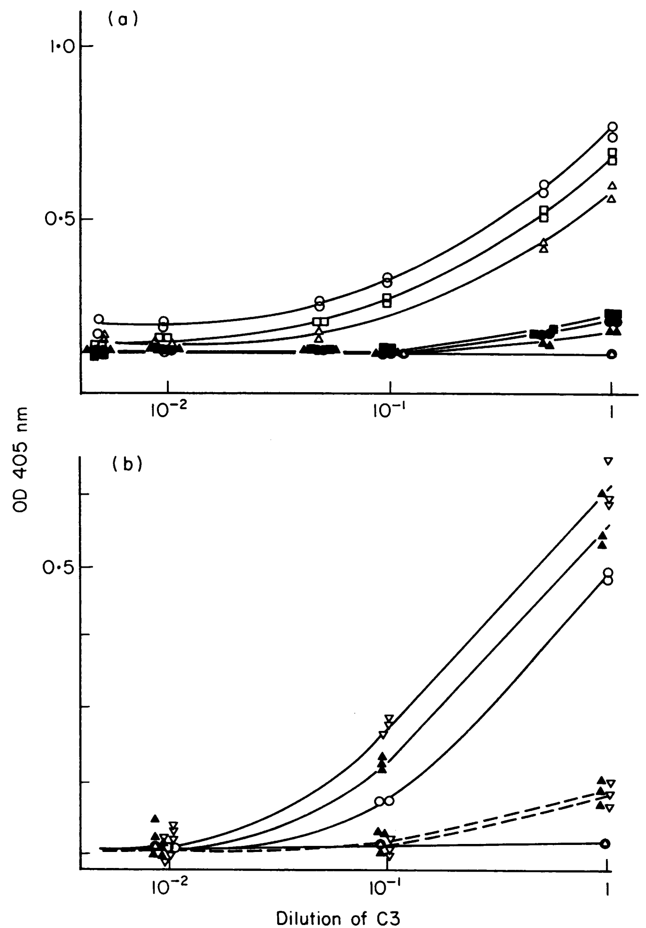

Figure 2. (a) Quantification of C3 in culture supernatants with ELISA: (○——○) human C3 (100 ng/ml); (△——△) C3 in primary culture medium (1 : 5 diluted); (□——□) C3 in culture medium of seven passages (undiluted). Blocking test of ELISA using the same samples: (●——●) human C3; (▲——▲) primary culture medium; (■——■) seven-passage medium; (○——○) control medium. (b) The effect of cycloheximide (5 μg/ml) on the release of C3: (○——○) human C3 (100 ng/ml); (▲——▲, ▽——▽) culture supernatants (five passages, undiluted); (▲– – –▲, ▽– – –▽) culture supernatants with cycloheximide (five passages, undiluted); (○——○) control medium.

DISCUSSION

Our results confirm that cultured human endothelial cells synthesize and release the third component of complement. The endothelial cells from the human scar described here have survived in culture for 13 passages without any specific conditions, although the growth rate has fallen slightly. While they have factor VIIIRAg specific for endothelial cells (Jaffe, 1977), and exhibit ring formations resembling capillary lumina (Tokunaga, Morimatsu & Nakashima, 1984; Folkman & Haudenschild, 1980), they lack Weibel–Palade bodies.

Cultured endothelial cells from several species have been shown to secrete several kinds of products: potent mitogens for connective tissue cells, endothelium-derived growth factor (EDGF) and platelet-derived growth factor (PDGF) (Gajdusek, DiCorleto & Schwarz, 1980; DiCorleto, 1984), prostacyclin (Jaffe, Hoyer & Nachman, 1973), anti-haemophilic factor antigen (VIIIRAg) (Gimbrone & Alexander, 1975), and type IV basement membrane collagen (Howard *et al.*, 1976). Little is known about the biosynthesis of complement components by cultured endothelial cells.

C3 is synthesized mainly by liver parenchymal cells, macrophages and also primary cultured fibroblasts. C3 isolated from

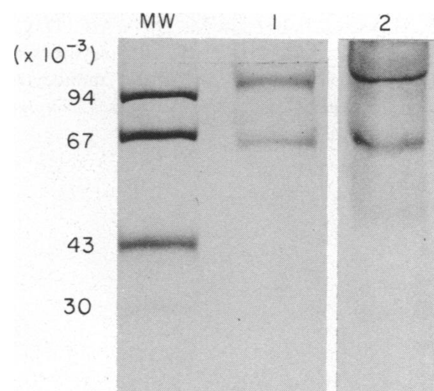

Figure 3. SDS-PAGE and autoradiography of secreted C3. Incubation with ^{35}S-methionine lasted 48 hr; autoradiography: 2 weeks at −70°. MW: molecular weight marker; Lane 1: stain; Lane 2: autoradiography.

plasma is a 187,000 MW glycoprotein composed of disulphide-linked α and β chains with molecular weights of 115,000 and 75,000, respectively (Bokisch, Dierich & Müller-Eberhard, 1976). C3 is initially synthesized as a single precursor, pro-C3 (Brade *et al.*, 1977), and the β chain is the NH_2-terminal segment of the pro-C3 molecule (Goldberger *et al.*, 1981). Misumi, Takami & Ikehara (1984) demonstrated that the cleavage of pro-C3 into the mature form with subunits takes place in the secretory vesicles just before secretion from hepatocytes, and all the secreted C3 they observed was of the mature processed form.

Quantitative determination of C3 produced by the cells was performed using ELISA. The endothelial cells secreted C3 at a rate of 250 ng/10^6 cells/5 days. The C3 secretion rate of cultured guinea-pig hepatocytes using ELISA, on the other hand, was 100 ng/10^6 cells/hr (Ramadori *et al.*, 1984). Cycloheximide reversively inhibited C3 production by the cells. These results show that cultured human endothelial cells synthesize and release C3 *in vitro*.

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