Complement Synthesis Influencing Factors Produced by Acute Myeloid Leukemia Blast Cells

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In a previous study, we found hypercomplementaemia in the sera of acute myeloid leukemia patients. In this study we show that the supernatants of mononuclear cells, derived from peripheral blood taken in the blastic phase, from patients with acute myeloid leukemia (CM-AML) increased the in vitro complement protein synthesis of HepG2 hepatocellular carcinoma cells. This effect of CM-AML was mediated by heat labile soluble factors and involved the synthesis of mRNA and protein. Inhibition experiments with anti-cytokine antibodies and immunoaffinity chromatography revealed that this effect of CM-AML is mostly mediated by IL-1 and IL-6. (Pathology Oncology Research Vol 1, No1, 54-59, 1995)

Key words: AML, complement factor B, C1-INH, C3, C4, IL-1b, IL-2, IL-6, TNF

Introduction

Hypercomplementaemia, or the elevation of the levels of over-all haemolytic activity of complement pathways and/or levels of complement components, above the upper limit of normal values, is frequently observed in malignant diseases.

In a previous study we have carried out measurements of complement activity in patients with acute myeloid leukemia (AML) and found that both the classical and the alternative pathway showed increased haemolytic activity and the haemolytic C4 titer and the serum level of C3 and factor B components were also increased as compared to those parameters in healthy blood donors. In the sera of acute leukemia patients, taken in the blastic phase, the haemolytic activity of the classical pathway and the level of factor B was significantly elevated compared to those parameters in remission.

As we have shown earlier, AML tumor cells produce factors that can increase complement protein synthesis by monocytes and cells of hepatocyte origin in vitro. In monocyte cultures the conditioned media of acute myeloid leukemia blast cells (CM-AML) increased C2 and factor B levels and decreased that of C1-esterase inhibitor (C1-INH). This study also shows that CM-AML increased factor B synthesis both at mRNA and protein level in HepG2 hepatocellular carcinoma cells. The complement synthesis enhancing potential of the supernatant of AML blast cells has also been demonstrated on fibroblasts: it substantially increased the factor B synthesis as measured by biosynthetic labelling, it did not affect, however, the production of C1-INH.

The main source of the synthesis of complement proteins in the human body are the hepatocytes of the liver, therefore we chose a well established hepatic cell line, HepG2 as a test system to further characterise the factor(s) produced by AML blasts. Here we report, that the factor B production increasing effect of CM-AML involves the synthesis of new mRNA and protein molecules and is mediated mostly by IL-1 and IL-6 released by the mononuclear cells of AML patients.
**Materials and Methods**

**Reagents**

Rabbit anti human C4 IgG was obtained from DAKO (Glostrup, Denmark); goat anti human factor B, C1-INH and C3 IgG and Calibrator 4 from Atlantic Antibodies (StIlwater, MN, USA). Heparin from Richter (Budapest, Hungary), HEPES, Cycloheximide, actinomycin D, amphotericin B and peroxidase labeled streptavidin were purchased from Sigma (St Louis, MO, USA). RPMI 1640 powder, FBS, glutamine and trypsin-EDTA from Flow Laboratories (Irvine, Scotland). Kanamycin from Serva (Heidelberg, Germany). Ficoll and CNBr activated Sepharose 4B from Pharmacia (Uppsala, Sweden). Uromon 75% from Bracco (Milano, Italy).

**Preparation of conditioned media**

Heparinized (5 U/ml) peripheral blood was taken with informed consent from three freshly diagnosed patients with acute myeloid leukemia (AML) in the Haematology Department of our Institute during the overt leukemic phase, when more than 90% of the cells in the periphery were tumor cells. According to FAB classification, patients K, E and P belonged to class M2, M4, and M5, respectively. Diagnosis was established on the basis of morphology, cytochemistry and surface markers. Mononuclear cells were separated by the standard Ficoll-Uronitro method (r=1.077 g/ml), washed three times with RPMI 1640. and then after adjusting the cell count to 10^6/ml they were cultured in RPMI 1640 containing 24 mM NaHCO3 and 20 mM HEPES, supplemented with 10% heat inactivated fetal bovine serum, glutamine, 20 μg/ml kanamycin and 2.5 μg/ml amphotericin B (referred below as culture medium) for three days at 37°C. Mononuclear cells from patient K were cultured in RPMI-1640, glutamine, antibiotics. The lack of serum proteins apparently did not influence the viability of cells. Then supernatants were harvested on the third day, centrifuged, sterile filtered (Minisart 0.22 μm, Sartorius, Goettingen, Germany) and stored in aliquots at -20°C until used.

**Conditioned media of the peripheral blood mononuclear cells of healthy adults (CM-PBMC)** were obtained and processed the same way as CM-AMLs.

**Cell cultures used for measurement of complement production**

The human hepatoblastoma cell line, HepG2, was maintained in RPMI 1640 containing 24 mM NaHCO3 and 20 mM HEPES, supplemented with 10% heat inactivated FBS, 2 mM Glutamine, 20 μg/ml kanamycin and 2.5 μg/ml amphotericin B and kept at 37°C in 5% CO2, in 250 cm2 culture flasks (Greiner, Frickenhausen, Germany).

For the experiments, the cells were trypsinized and 200 μl cell suspension was seeded into the wells of 96-well tissue culture plate (Becton Dickinson, Lincoln Park, NJ, USA) at a density of 2-3 x 10^4 cells per ml. Cultures were treated on the next day with CM-AMLs (1:2 dilution with raw culture medium) for 24 hours then supernatants harvested and stored at -20°C until tested for the level of complement proteins.

In the case of long term experiments, cells were washed twice with culture medium at the end of the first 24 hrs culture period and then cultured for further 24 hrs and supernatants were then harvested.

Cultures were inspected before and at the termination of experiments through an inverted light microscope.

**Assay of complement proteins**

Factor B, C1-INH, C3 and C4 concentrations in the supernatants of HepG2 cultures were measured by double polyclonal sandwich ELISA. Briefly, flat bottomed 96 well microwell plates (Greiner) were coated by the respective antibody preparation (IgG fraction), blocked then standard (serial dilutions of Calibrator 4) and the samples were applied on the plate. The captured antigen was detected by the same antibody labeled with horse radish peroxidase. In the case of C1-INH biotinylated antibody and streptavidin-peroxidase conjugate was used. The colorgenic reaction was performed with TMB/ureaperoxide or OPD/ureaperoxidase substrate (measured at 450 nm/620 nm or 492 nm/620 nm, respectively, on an Anthos II ELISA plate reader). The detection limit of the tests were 2 ng/ml for factor B, 100 pg/ml for C1-INH, 10 ng/ml for C3 and 2 ng/ml for C4.

**Heat inactivation of CM-AMLs**

Aliquots of CM-AMLs were submerged in boiling water for 10 minutes, the spun down, sterile filtered and diluted to 1:2 with culture medium and added to HepG2 cultures with suitable controls.

**Cytokine inhibition experiments**

Anti-cytokine antisera or purified IgG antibodies were added to CM-AMLs, incubated for 30 minutes at 37°C then brought to HepG2 cell cultures. Supernatants were harvested next day and factor B levels determined.

Anti-cytokine antisera or antibodies were kindly provided or purchased as follows: rabbit anti-human interleukin-2 IgG (<1μg/ml neutralises 1 U/ml IL-2 in biological assay, 1 mg/ml, used in 1:100) (Boehringer Mannheim); mouse monoclonal anti-human interleukin 6 IgG (20 μg neutralises 1 U IL-6 in biological assay, 1 mg/ml, used in 1:10.000) (Biosource International); goat anti-human interleukin-1-beta IgG (10 μg/ml neutralises 50 μg/ml IL-1 in biological assay, 1 mg/ml, used in 1:1000) (British Biotechnology); rabbit anti-human tumor necrosis factor antiserum 10.000 neutralising U/ml, used in 1:1000). Immunogenetics; anti-TNF rabbit antiserum, (Dr Michael Kirschfink, Johannes Gutenberg Universität, Heidelberg, Germany); rabbit anti-human...