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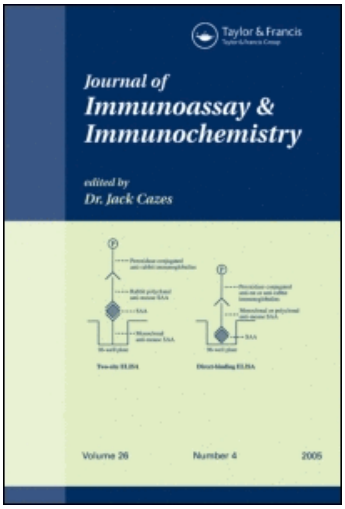

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Association of CD2 with Fibrinogen in Human Plasma: Depletion of the Soluble E-Receptor in Blood Clotting

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Abstract: The soluble E-receptor (SER) of lymphocytes that is related to CD2 was detected in human plasma and serum using immunoelectrophoresis with sheep antiserum. All plasma samples (n = 18) demonstrated reactivity with antiserum, whereas the reactivity of the corresponding sera remained low or undetectable. The depletion of SER in clotting is associated with fibrinogen, as shown by crossed-affinity immunoelectrophoresis with antisera to plasma proteins. The SER-associated fibrinogen was purified and analysed by the SDS-polyacrylamide gel electrophoresis and immunoblotting. A band close to 66 kDa was detected with monoclonal antibodies to CD2. The association of CD2 and other soluble receptors with fibrinogen via domains is suggested. It is recommended that the fresh plasma, not serum, should be used to study circulating receptors because coagulation may appreciably diminish their physiological level in blood samples.

Keywords: CD2, Fibrinogen, Soluble E-receptor, Alpha-2-macroglobulin, Clotting

INTRODUCTION

The cellular receptors are present in blood in a soluble form as a result of cleavage or exocytosis.[1] The cell-surface glycoprotein CD2 (T11, or

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E-receptor to sheep erythrocytes) is implicated in adhesion, signaling, and activation of T-lymphocytes and NK cells.[2,3] The soluble CD2 may inhibit the T-cell activation and cell-to-cell adhesion *via* CD2/LFA3 interaction;[4] therefore, it rather should be scavenged. Earlier, the authors demonstrated the association of CD2 with an α2-macroglobulin (α2M) in the plasma of patients with gastric cancer. The complex was purified and partially characterized.[5]

To determine the level of the soluble E-receptor (SER) in different pathological conditions, sheep antiserum to the E-receptor (ERSA) was prepared by immunization of the sheep with the E-receptor coated autologous erythrocytes.[6] An elevated serum level of SER and CD2 was found in HIV-1 positive and AIDS patients.[7,8] We observed an increased serum level of SER in 60% of gastric carcinoma patients and in only 5% of donors.[9] But, unlike the serum, the blood plasma was found to exhibit a high ERSA-reactivity in all the subjects investigated. The present study demonstrates the association of SER with plasma fibrinogen, which explains the loss of SER in serum after clotting.

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EXPERIMENTAL

Antisera

ERSA was produced as described by Mendes et al.,[6] with some modifications.[5] Rabbit antisera to such human plasma proteins as fibrinogen, haptoglobins (Dakopatts, Denmark), fibronectin, sheep antiserum to IgG (H+L chains) (Institute of Molecular Pathology, Estonia), apo-B100 and apo-A1 lipoproteins (Orion Diagnostica, Espoo, Finland) were used.

Preparation of the Mononuclear Extract

The mononuclears were isolated from donor blood and the E-receptor was extracted with Triton X-100 in the presence of proteinase inhibitors.[5]

Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis was performed by the Laurell technique in 1.2% agarose with a veronal buffer (pH 8.5) containing 50 μL of ERSA per mL of the gel.[10] Six μL of serum or one μL of plasma were applied to the gel.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis was carried out as described by Weeke.[11] The conditions were as follows: the first (horizontal) direction, 10 v/cm for

4 h, the second (vertical) direction, 2 v/cm, overnight. The upper gel contained 50 μL of ERSA per mL (Figure 1).

Crossed-Affinity Immunoelectrophoresis

Crossed-affinity immunoelectrophoresis was performed in the same way as crossed immunoelectrophoresis, but the lower first-direction-gel contained the non-immune rabbit serum in the control or the rabbit monospecific antiserum to fibrinogen in the test, both 60 μL/mL. Besides, immunoelectrophoresis with antiserum to fibronectin (14 μL/mL), apolipoprotein B100 (100 μL/mL), apolipoprotein A1 (30 μL/mL), haptoglobins (100 μL/mL) or IgG (120 μL/mL) was performed. The upper gel contained ERSA (Figure 2). Conversely (Figure 3), the lower gel contained the nonimmune sheep serum or ERSA (both 120 μL/mL) in the control or test, respectively. The upper second-direction-gel contained antiserum to fibrinogen (8 μL/mL).

In all the above methods, the agarose gels were washed and stained with Coomassie R-250.

Purification of the E-Receptor-Associated Fibrinogen (ER-Fibrinogen)

20 mL of the whole blood was collected in plastic tubes containing 2 mL of a 3.8% trisodium citrate/2.5% 6-aminocaproic acid solution and 4 mg of a crude soybean trypsin inhibitor (Reanal, Hungary). After centrifugation, plasma (12 mL) was collected and 1.2 mL of $BaSO_4$ suspension (30 mg/mL) in 0.8 M $BaCl_2$ solution was slowly added with stirring. The mixture was stirred for 1 h and 0.64 mL of 1.5 M Na_2SO_4 was added to remove barium ions. After centrifugation at 5,000 g, the supernatant (13 mL) was twice extracted with 6 mL of hexane to remove lipids. The protein solution was centrifuged at 15,000 g for 40 min and dialysed against 0.05 M sodium acetate buffer, pH 5.0. The solution was centrifuged (40 min, 15,000 g) and an equal volume of 0.05 M sodium acetate/4 M NaCl buffer, pH 5.0, was

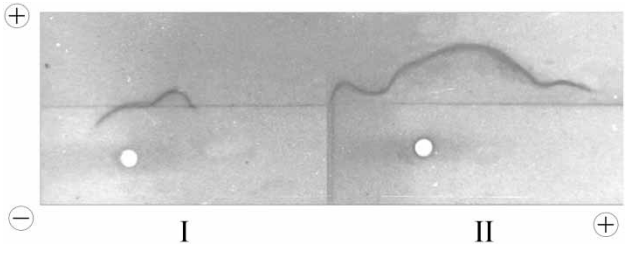

Figure 1. Crossed immunoelectrophoresis. Samples from patient with gastric cancer: I - serum, II - plasma. The upper gel contained ERSA.

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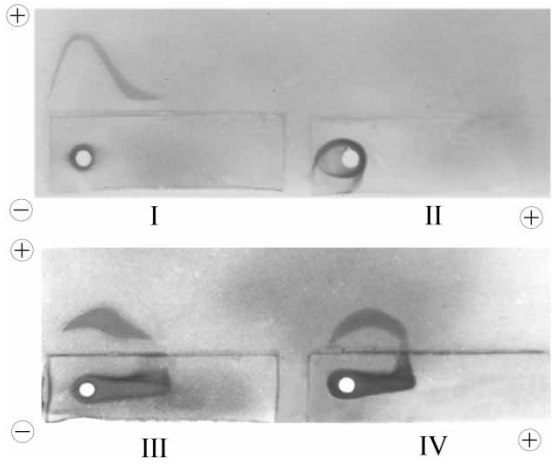

Figure 2. Crossed-affinity immunoelectrophoresis. The sample is the plasma of donor. Lower gels contained: nonimmune rabbit serum (I), rabbit antiserum to fibrinogen (II), antiserum to fibronectin (III) and antiserum to apo-B lipoprotein (IV). The upper gel contained ERSA everywhere.

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slowly added to the supernatant with stirring. The mixture was centrifuged (40 min, 15,000 g). The supernatant (760 units at A_{280} nm) was applied to the column with 36 mL of the Cibacron Blue F3-GA agarose (Kemotex, Estonia) equilibrated with 0.05 M sodium acetate/2 M NaCl buffer, pH 5.0. The unbound proteins were washed with the buffer. The ERSA-positive fraction was eluted with 0.05 M Tris HCl/1 M-KSCN buffer, pH 8.0. The fraction was dialysed against 0.07 M trisodium citrate and concentrated by ultrafiltration. The fraction was dialysed against 0.05 M sodium acetate (pH 5.0) and centrifuged. The supernatant was applied to the column with Cibacron Blue agarose (12 mL) which was equilibrated with 0.05 M sodium acetate, pH 5.0. The column was washed with 0.05 M sodium acetate/2 M NaCl, pH 5.5. The ERSA-positive fraction was eluted with 0.05 M Tris HCl/1 M KSCN, pH 8.0. The product was dialysed against the buffer used

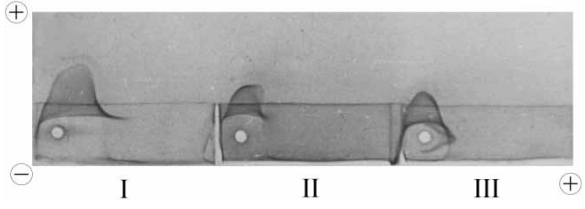

Figure 3. Crossed-affinity immunoelectrophoresis. The sample is the plasma of donor. Lower gels contained: buffer (I), nonimmune sheep serum (II), ERSA (III). The upper gel contained the antiserum to fibrinogen.

for size-exclusion chromatography (0.08 M sodium phosphate/0.32 M NaCl, pH 7.3), and then concentrated. The fraction (1 mL, 47 units at A_{280} nm) was applied to the column (2.4 × 55 cm) with TSK-Gel Toyopearl HW-60 (Fine), (Toyo Soda, Japan). The fibrinogen fractions were analysed by polyacrylamide gel electrophoresis (PAGE), and rocket immunoelectrophoresis using antisera to plasma proteins. The fractions were purified on immunosorbents with immobilized antibodies to immunoglobulins, haptoglobins, and apolipoprotein B100 as described earlier.[5] Finally, the heparin-binding proteins were removed on heparin-agarose (Kemotex, Estonia).[12] In all steps, the fractions were controlled with ERSA and antiserum to fibrinogen by rocket immunoelectrophoresis.

The E-receptor-associated α2-macroglobulin (ER-α2M) was purified as described earlier.[5]

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Polyacrylamide Gel Electrophoresis

A discontinuous PAGE was performed in 5% gel.[5] Sodium dodecyl sulfate (SDS) gel electrophoresis was performed in a 4–20% linear gradient of PAG according to Laemmli.[13] The samples in 2% SDS/6 M urea/0.5 M dithiothreitol/0.001% Bromophenol Blue/0.0625 M Tris HCl, pH 6.8 were incubated at 37°C for 1 h and applied onto the gel.

Immunoblotting

Immunoblotting was performed as described earlier, using the following mouse monoclonal antibodies to CD2: T11, clone SFCI3Pt2H9 (Beckman Coulter, USA), and LT-2b (Institute of Immunology, Moscow).[5] The monoclonal antibody to potato virus (National Institute of Chemical Physics & Biophysics, Estonia) was used as a negative control.

RESULTS

All plasma samples (n = 18) contained the ERSA-reacting antigen (E-antigen), as shown by rocket immunoelectrophoresis. The clotting of blood or incubation of plasma with calcium ions led to a significant reduction or disappearance of the peak. The difference between serum and plasma was shown by crossed immunoelectrophoresis: the fused peaks with a wide area were formed for plasma and only a minor amount of the precipitate for serum (Figure. 1). The E-antigen was associated mainly with fibrinogen because it had been completely precipitated by antiserum to fibrinogen, as shown by crossed-affinity immunoelectrophoresis (Figure 2, I, II). Conversely, ERSA decreased the peak of fibrinogen only partly (Figure 3). Control

non-immune sheep serum aggregated the fibrinogen during electrophoresis in the first direction, thereby decreasing the size of the peak in the upper gel. The antiserum to fibronectin and apolipoprotein B100 exhausted the E-antigen partially (Figure 2, III, IV). A similar effect was observed for antisera to haptoglobins, apolipoprotein A1 and IgG (not shown).

The fractions of ER-fibrinogen were isolated by chromatography on Cibacron Blue agarose and further on the TSK gel HW-60 (Figure 4). The negligible amounts of admixtures of low-density lipoproteins, immunoglobulins and haptoglobins were removed by immunoadsorbtion. Fibronectin and other heparin-binding proteins were removed on heparin-agarose. The final homogeneous product in the reduced SDS-PAGE migrated as Aα-, Bβ-, and γ-chains typical of fibrinogen but, in the region near 66 kDa, a wider band was detected (Figure 5, I, b). Both the ER-fibrinogen and ER-α2M under reducing conditions revealed the bands with an apparent molecular weight (MW) of about 66 kDa which were closely located to that of an Aα-chain of fibrinogen and bovine serum albumin (Figure 5). For non-reduced samples, the bands were weakly visible (Figure 5, III). A specific reaction for the bands with an MW of approximately 66 kDa was revealed by reducing SDS-PAGE-immunoblotting with a monoclonal antibody T 11 (Figure 5, II). A similar pattern was observed for a monoclonal antibody LT-2b, but an irrelevant control monoclonal antibody to potato virus had no effect in immunodetection. The nonreduced ER-fibrinogen and ER-α2M did not reveal any bands in immunoblotting with CD2-specific monoclonal antibodies. Thus, the fibrinogen fraction associated with the CD2 receptor was isolated from human plasma.

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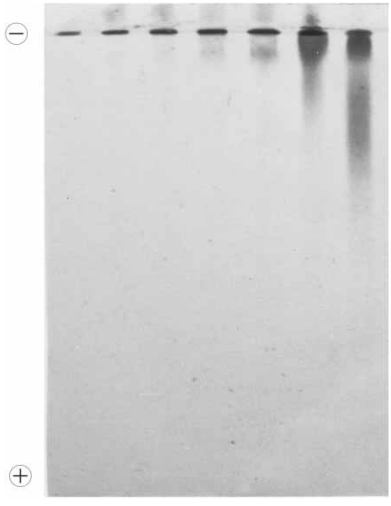

Figure 4. Discontinuous 5% PAG electrophoresis of fibrinogen fractions purified on TSK-GEL HW-60 (elution in the interval 107–124 mL, the volume of TSK-gel was 249 mL).

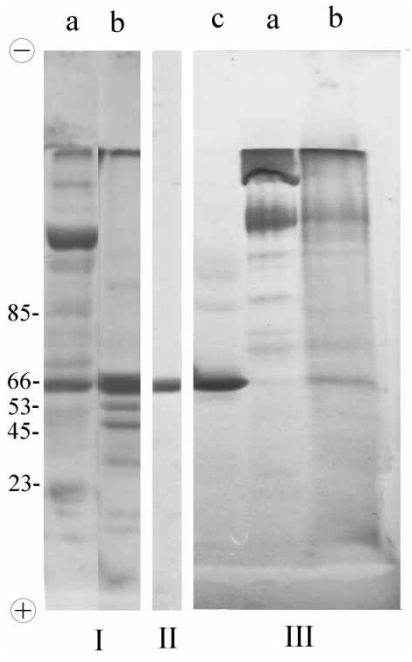

Figure 5. SDS-PAGE and immunoblotting. I – reduced with dithiothreitol samples, III – nonreduced samples: ER-α2M (a), ER-fibrinogen (b), BSA (c). The calibration in kDa: L and H-chains of human IgG (23 and 53, respectively), egg albumin (45), BSA (66), the reduced α2M-trypsin complex (85). II – transferred onto a nitrocellulose sheet reduced ER-fibrinogen; immunodetection with a monoclonal antibody T11.

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DISCUSSION

The E-antigen was rarely revealed in donor sera by ERSA-rocked immunoelectrophoresis. When plasma was used instead of serum, a significantly higher reactivity in all the probes was observed. Fibrinogen is involved in the disappearance of the E-antigen in blood clotting, as shown by crossed-affinity electrophoresis with different antisera. ERSA did not precipitate the whole fibrinogen (Figure 3), but precipitated and removed the fraction of the purified ER-fibrinogen. Earlier, the authors isolated the complex of α2M with CD2 and compared it with α2M.[5] The purified ER-fibrinogen and ER-α2M were analysed by ERSA-rocket-line immunoelectrophoresis and ERSA-tandem-crossed immunoelectrophoresis with an extract of peripheral blood mononuclears (data not shown). The extract contained a negligible amount of fibrinogen and no α2M at all. The fused peaks of the ER-fibrinogen with ER-α2M and antigens of the extract were revealed in immunoelectrophoresis that may indicate the presence of a common antigen. This viewpoint was further confirmed by SDS-PAGE. Both the

ER-fibrinogen and ER-α2M contained the component with an MW close to that of bovine serum albumin (Figure 5). In size, the component corresponded to the T-lymphocyte antigen Ts_1, which was earlier isolated from the serum of a patient with a renal failure and is apparently involved in the sheep red cell rosette formation.[14] The positive reactivity of this component with monoclonal antibodies in immunoblotting confirms the presence of CD2 (Figure 5).

The heterogeneity of the E-antigen was shown by crossed immunoelectrophoresis (Figure 1), as well as by size-exclusion chromatography on the TSK-gel. The different electrophoretic mobility and molecular forms of serum SER have been established by other researchers as well.[14,15] The detected heterogeneity of the E-antigen may be explained by the following. ERSA demonstrates a broader specificity and may react, not only with SER, but also with other circulating receptors which are released from the cells to plasma. ESRA was produced by immunization of the sheep with autologous erythrocytes coated by human mononuclear receptors, where the E-receptor appeared to be the main component. The antibody response was probably induced by the immunogenic determinants of the E-receptor exhibiting a cross-reactivity with determinants of other receptors. Besides, a free form of SER or the form associated with other proteins may be present in plasma. This may explain the overlapping reactivity of antigens forming broad fused precipitates with ERSA, and their heterogeneity.

The molecular substructures drawn in CD2-fibrinogen binding deserve further study. CD2 belongs to adhesion molecules of the immunoglobulin superfamily.[3] Both the fibrinogen and α2M expose multiple regions responsible for different binding interactions.[16–18]

The fibrin-related components, such as D-dimer, a fibrin monomer, soluble fibrin complexes, or fibrin(ogen) degradation products (FDP), are frequently present in the plasma of patients with malignancy due to the activation of hemostasis and fibrinolysis in disseminated intravascular coagulation. FDP remains in the serum after clotting. In addition, a good correlation between FDP levels in serum and plasma samples was established.[19,20] We frequently observed the presence of the remaining E-antigen in the sera of gastric carcinoma patients in immunoelectrophoresis. The antiserum to fibrinogen was also found to exhaust the E-antigen from serum. Probably, the FDP-associated E-antigen is revealed in the sera of patients with cancer that is a consequence of an abnormal hemostasis and fibrinolysis.

The mechanism of SER depletion during clotting needs to be clarified. SER may be additionally captured during the aggregation and conversion of fibrinogen to fibrin. Methylamine (10 mM, an inhibitor of transglutaminase cross-linking), as well as dithiothreitol or heparin, abrogate the depletion of the E-antigen in the plasma induced by calcium ions. We observed a high, but vague, peak for plasma with citrate and heparin in ERSA-rocket immunoelectrophoresis. The peak was hardly recognizable when the fresh blood without anticoagulants was centrifuged and analysed at once. But, the

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treatment of plasma with dithiothreitol results in the appearance of a contrasting peak. A cryptic disulfide-bound form of the E-antigen may be present in circulation. This is in accordance with the results of SDS-PAGE and immunoblotting, where the CD2-positive bands were detected for the reduced ER-fibrinogen and ER-α2M.

The serum samples are conventionally used in many laboratories. However, the manipulation with blood *in vitro* can induce a cascade of enzymatic and nonenzymatic reactions, the shedding of cellular receptors, or molecular adhesion. In a wide sense, the data obtained on the detection of circulating receptors and their ligands in serum may not truly reflect patho-physiological conditions or may be misconstrued.

CONCLUSIONS

Blood clotting or incubation of plasma with calcium ions results in the loss of SER. The loss of SER in coagulation is associated with fibrinogen. The fraction of the ER-fibrinogen isolated from plasma contained a component with an MW of approximately 66 kDa, which was immunodetected with monoclonal antibodies to CD2. The authors assume that other soluble receptors may form complexes with fibrinogen as well, and clotting may diminish their level in blood samples. The use of the fresh plasma should be preferred to determine the level of circulating receptors and to study their patho-physiological role in human.

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ABBREVIATIONS

SER	soluble E-receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
ERSA	sheep antiserum to the E-receptor
E-antigen	ERSA-reacting antigen
ER-fibrinogen	E-receptor-associated fibrinogen
ER-α2M	E-receptor associated α2-macroglobulin
A	absorbance
FDP	fibrin(ogen) degradation products

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