The 95000- M_r gelatin-binding protein in human serum and plasma

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A gelatin-binding 95000- M_r protein was detected in human serum and plasma by immunoblotting using antibodies against the 95000- M_r gelatin-binding protein, a major secretory component of cultured adherent human monocyte/macrophages. Serum and plasma were prepared by incubating blood at 4, 22 or 37°C for different periods of time, and gelatin-binding proteins were isolated from 200 μ l portions by gelatin–Sephadex affinity chromatography. The bound material was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In protein-stained gels, fibronectin and some minor polypeptides were seen, but not the 95000- M_r protein. In immunoblotting of identical serum samples the antibodies detected apparently two closely spaced polypeptide bands at M_r 95000, and in plasma samples a single band at the position of the faster-migrating one of the two above-mentioned bands. The immunoperoxidase reaction was stronger when serum and plasma were prepared by incubating for longer periods of time (up to 8 h) or at higher temperatures (up to 37°C). In samples made from plasma, the immunoperoxidase reactions were weaker than in those from serum, indicating a lower quantity of the protein. The results suggest that the 95000- M_r protein is released from monocytes and granulocytes during the incubation of blood and, more likely, when they possibly interact with the blood clot and may become adherent.

Human monocyte/macrophages produce an exceptionally large variety of substances involved in the host defence systems, e.g. in inflammatory conditions (Nathan *et al.*, 1980; Lasser, 1983). These include, among others, complement components, proteolytic enzymes and both activators and inhibitors of proteinases. In a previous study we have shown that cultured adherent human macrophages and U-937 pro-monocytic cells produce as their major secretory component a 95000- M_r gelatin-binding protein (Vartio *et al.*, 1982a,b), which was also found in blood granulocytes, but not in any other cell type in blood (Vartio *et al.*, 1985). This protein had no immunological cross-reactivity with other gelatin-binding proteins produced by cultured cells, namely fibronectin and a 70000- M_r polypeptide (Vartio & Vaheri, 1981; Vartio *et al.*, 1982a,b).

The present study describes the detection of a 95000- M_r gelatin-binding protein in human serum and plasma by using immunoblotting and anti-

bodies against the 95000- M_r protein produced by cultured human macrophages. The results suggest that the protein is released from the monocyte/macrophages and/or granulocytes when, possibly, they interact with the blood clot.

Materials and methods

Plasma and serum samples

Whole blood was obtained from healthy donors and immediately divided into 5 ml aliquots. To prevent clotting, half of the tubes contained 100 μ l of 19% (w/v) sodium citrate (final concn. 0.38%). Tubes with or without sodium citrate were then incubated at 4, 22 or 37°C for 2, 4, 6 or 8 h. After incubation the tubes were centrifuged at 1200 rev./min (r 15 cm) for 10 min to isolate serum and plasma from blood cells.

Affinity chromatography

Serum and plasma samples (200 or 400 μ l) were treated with 80 μ l of 50% (v/v) gelatin–Sephadex in NaCl/ P_i , incubated in an end-over rotator for 2 h, washed with NaCl/ P_i and mixed with 40 μ l of

Abbreviations used: SDS, sodium dodecyl sulphate; NaCl/ P_i , 0.01 M-sodium phosphate/0.14 M-NaCl, pH 7.4.

Laemmli's (1970) sample buffer to analyse the bound proteins by SDS/polyacrylamide-gel electrophoresis (see below). Before gelatin-Sepharose chromatography, the serum and plasma samples were treated with unsubstituted Sepharose 4B to remove most of the non-specifically binding proteins. In control experiments unsubstituted Sepharose 4B was used instead of gelatin-Sepharose.

SDS/polyacrylamide-gel electrophoresis

This was performed as described by Laemmli (1970), with vertical slab gels. The acrylamide concentration was 3.3% in the spacer gel and 6% in the separating gel. The samples were reduced with 10% (v/v) 2-mercaptoethanol in Laemmli's (1970) sample buffer. After electrophoresis, the gels were either stained with Coomassie Brilliant Blue or used in immunoblotting (see below). Commercially obtained M_r markers (Pharmacia, Uppsala, Sweden) were used.

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets

Immunoblotting was performed as described by Towbin *et al.* (1979). In this procedure, polypeptides separated by SDS/polyacrylamide-gel electrophoresis were transferred electrophoretically to nitrocellulose sheets (type I HAWP filter; Millipore, Bedford, MA, U.S.A.). As a modification of the original method (Towbin *et al.*, 1979), the transferred proteins were located by using reversible heparin/toluidine staining (Vartio *et al.*, 1982a,b).

Other methods

Human blood monocyte/macrophages were isolated and cultured as described by Hovi *et al.* (1976). The 95000- M_r protein was purified by gelatin-Sepharose chromatography from serum-free growth medium of cultured human monocyte/macrophages and antiserum against it was prepared and characterized as described previously (Vartio *et al.*, 1982a,b). Granulocytes were isolated from the buffy-coat fractions of human blood as described by Bøyum (1968). The granulocytes were extracted with 0.5% Triton X-100 in 5mM-Tris/HCl, pH 7.5, and the 95000- M_r protein was isolated by gelatin-Sepharose chromatography as described by Vartio *et al.* (1985). The antiserum was used in the immunoblotting experiments at a dilution of 1:100 in NaCl/P_i. In control immunoblotting, the anti-(95000- M_r protein) serum was replaced by non-immune rabbit serum.

CNBr-activated Sepharose 4B was obtained from Pharmacia. Gelatin (porcine type I; Sigma) was coupled to activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Results

Detection of the 95000- M_r protein in serum and plasma

Human serum and plasma from the same blood sample were prepared by incubating blood, with or without sodium citrate as an anticoagulant, at different temperatures (4, 22 and 37°C) for 2, 4, 6 or 8 h. The gelatin-binding proteins from the resulting serum and plasma were then isolated and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. Fig. 1 shows SDS/polyacrylamide-gel electrophoresis of the gelatin-binding proteins from 200 μ l of serum and plasma prepared by incubating blood at 37°C for 4 h. In addition to fibronectin, several minor components were seen

Fig. 1. SDS/polyacrylamide-gel electrophoresis of gelatin-binding proteins from human serum and plasma

Aliquots (200 μ l) of serum and plasma, both prepared by incubating human blood for 4 h at 37°C, were treated with gelatin-Sepharose and the bound material was analysed in a SDS/6%-(w/v)-polyacrylamide-gel electrophoresis as described in the Materials and methods section. Track 1 shows the gelatin-binding material from serum (S) and track 2 that from plasma (P). The position of 95000- M_r protein is indicated by '95K'. Abbreviation used: FN, fibronectin.

(Fig. 1). These proteins were presumably not fibronectin fragments, since they did not react with anti-fibronectin antibodies on immunoblotting (T. Vartio, unpublished work). No attempt for further characterization of these components was performed.

The 95000- M_r polypeptide could not be positively identified in this protein-stained gel. Fig. 2 shows immunoblotting of identical samples with antiserum raised against the 95000- M_r macrophage protein (tracks 1 and 2). The immunoperoxidase reaction at the 95000- M_r position is clearly stronger in the sample made from serum than in that made from plasma. Furthermore, the immunoperoxidase reaction in the serum sample appeared as a broad zone that was apparently composed of two closely spaced bands (track 1, Fig. 2), whereas that in plasma sample formed only one band (tracks 2 and 3, Fig. 2), co-migrating with the faster one of the two bands mentioned above. This is seen more clearly in the immunoperoxidase reaction in track 3, in which the sample was made from double the amount (400 μ l) of the same plasma used in track 2. The gelatin-binding proteins from the Triton X-100 extract of human blood granulocytes (the 95000- M_r protein) and

from the growth medium of cultured human blood macrophages (fibronectin and the 95000- M_r protein) are shown in protein-stained tracks 4 and 5 respectively. As a control, identical samples were immunoblotted with the anti-(95000- M_r protein) serum (tracks 6 and 7 respectively).

The gelatin-binding proteins from serum and plasma samples, prepared by incubating portions of blood at 37°C for 2, 4, 6 or 8 h, were also analysed by immunoblotting. Fig. 3 shows such an experiment performed with serum samples. It can be seen that the strength of the immunoperoxidase reaction increased as a function of time, indicating the increase in the quantity of the protein in samples prepared after longer incubations. When similar samples were made at lower temperatures (22 or 4°C) as well as from portions of plasma, the immunoperoxidase reactions were weaker or absent (results not shown).

When 3 μ l of total serum or plasma was electrophoresed and immunoblotted, no immunoperoxidase reaction was detected by using anti-(95000- M_r protein) serum at the dilution of 1:100

Fig. 2. Immunoblotting of the gelatin-binding proteins from human serum, plasma and culture medium of macrophages

Samples of the gelatin-binding material from serum and plasma identical with those shown in Fig. 1 were immunoblotted and treated with anti-(95000- M_r protein) serum as described in the Materials and methods section. Track 1, the immunoperoxidase reaction in the serum sample (S); track 2, that in plasma sample (P); and track 3, the same with double the amount of sample material. Tracks 4 and 5 show a protein-stained gel [SDS/6%-(w/v)-polyacrylamide-gel electrophoresis] of the gelatin-binding proteins from human granulocyte extract and from growth medium of cultured human macrophages, and tracks 6 and 7 show immunoperoxidase reactions after anti-(95000- M_r protein) serum treatment of identical samples on immunoblotting. Abbreviation used: FN, fibronectin.

Fig. 3. Immunoblotting of the gelatin-binding proteins from sera made after various periods of incubations of blood

Portions of blood were incubated without anticoagulant at 37°C for 2, 4, 6 or 8 h. Sera were separated from blood cells and the gelatin-binding proteins were isolated from them and immunoblotted by anti-(95000- M_r protein) serum as described in the Materials and methods section. Note the increasing intensity of the immunoperoxidase reaction as a function of the time (indicated at the bottom of the Figure).

(results not shown). Nor were immunoperoxidase reactions detected in control experiments when non-immune rabbit serum was used or when unconjugated Sepharose 4B was used instead of gelatin-Sepharose.

Discussion

The present paper shows that a protein immunologically cross-reactive with the major secretory polypeptide produced by cultured human macrophages (M_r 95000; Vartio *et al.*, 1982a,b) can be detected in human serum and plasma. The protein has also been detected in blood granulocytes, but not in other blood cells (Vartio *et al.*, 1985). The evidence presented is based on the partial purification of the protein by gelatin-Sepharose from serum and plasma and on the detection of it in immunoblotting with antibodies raised against the 95000- M_r macrophage protein.

Immunoperoxidase reaction in the gelatin-binding material at the position of 95000 M_r was stronger when serum was prepared by clot formation at 37°C than when lower temperatures were used. The reaction was also stronger the longer period of time blood had been incubated in the preparation of serum and plasma samples (Fig. 3). In the samples made from plasma, the reactions were weaker than in corresponding serum samples (Fig. 2, tracks 1 and 2). The stronger immunoperoxidase reaction in the serum samples suggests the presence of a greater amount of the protein, although the methods used are not quantitative. In view of the undetectability of the M_r 95000 protein in protein-stained gels (Fig. 1) or in immunoblotting of 3 μ l of the whole serum and plasma, it can be estimated that its concentration is very low, perhaps only a few micrograms per ml.

These findings suggest that macrophages and granulocytes release the protein during the incubation and preferably at a physiological temperature. It is possible that monocytes and/or granulocytes interact with the clot, become adherent and concomitantly secrete the 95000- M_r protein. This would be in line with the previous studies in which only the adherent and spread cultured monocyte/macrophages and U 937 pro-monocytic cells, unlike the U 937 cells grown in suspension, were found to secrete the protein (Vartio *et al.*, 1982a,b). However, the possibility that the protein is released as a result of cell destruction *in vitro* cannot totally be excluded. If this were true, it would have been expected that the immunoperoxidase reactions would have been identical in both serum and plasma samples.

In serum-derived samples the immunoperoxidase reaction formed a broad band, apparently representing a doublet of two closely spaced bands, whereas that in plasma samples was a narrow single band co-migrating with the lower band of the doublet mentioned above. The explanation could be that the 95000- M_r protein in plasma was derived only from macrophages, whereas the bands detected in serum were formed from the proteins produced by both macrophages and granulocytes. This interpretation corresponds to a previous study in which the 95000- M_r protein detected in human blood granulocytes had somewhat slower electrophoretic mobility than that produced by macrophages (Vartio *et al.*, 1985), as shown in Fig. 2, tracks 4 and 5. Alternatively, the slower-migrating band may represent a precursor form of the 95000- M_r macrophage protein, although no such a protein has been detected in cell-culture experiments (Vartio *et al.*, 1982a,b).

In spite of the present data obtained *in vitro*, it is not known whether the 95000- M_r protein is a normal component of blood *in vivo*. It may, however, be possible to detect it on occasions when granulocytes and monocytes become adherent and the proteins they secrete have access into the blood. More detailed studies are, however, needed to elucidate the appearance of the 95000- M_r protein in serum and plasma, or whether the protein could also be used as a marker of certain pathological or physiological conditions, e.g. those in which blood clotting takes place.

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