Isolation and Identification of a Human Serum Fibronectin-like Protein Elevated during Malignant Disease

Robert G. Parsons, Heather D. Todd, and Robert Kowal

ABSTRACT

A human DNA-binding protein, designated MAD-2, has recently been found to be elevated in the serum from patients with malignant diseases. MAD-2 has been purified approximately 500-fold from peritoneal and pleural fluids collected from cancer patients. Immunodiffusion studies have indicated that MAD-2 is immunochemically identical to human plasma fibronectin. The purified material has been resolved by sodium dodecyl sulfate gel electrophoresis into two major protein chains with molecular weights in the range of 200,000 to 210,000 in either the presence or absence of disulfide bond-reducing agents. These results suggest that MAD-2 is a fibronectin fragment which has been generated through proteolysis. A quantitative assay system capable of detecting ng quantities of MAD-2 has been developed and used to verify the presence of elevated MAD-2 levels in DNA-binding protein fractions isolated from the serum of individuals with malignant diseases.

INTRODUCTION

A variety of studies have been aimed at identifying unique proteins in body fluids which would be useful markers of neoplasia. One specific approach has utilized DNA-cellulose chromatography as a means of fractionating human serum proteins. This approach has involved the comparison of DNA-binding protein fractions from normal sera to analogous fractions from sera of individuals with malignant disease, attempting to visualize unique species in the sera of cancer patients. Two human serum DNA-binding proteins have been observed to be present at elevated levels in the sera of individuals with malignant disease (7, 12). The first detected malignant disease-associated serum DNA-binding protein, termed C3DP, was isolated and identified as a fragment of complement component C3 (13). An assay system was developed for C3DP, and preliminary clinical studies have indicated its usefulness as a tumor marker (14, 16). A second protein, designated MAD-2, was recently reported to be present in a fraction eluted with 0.6 M NaCl from DNA-cellulose (12) and was shown to be distinct from C3DP. Elevated MAD-2 levels have been reported to be present in the sera from a high percentage (69%) of individuals with carcinomas of various primary sites.

This report describes the isolation and identification of MAD-2. The development of a radioimmunoassay system for MAD-2 is described, and preliminary results of quantitative determinations on serum DNA-binding protein fractions from normal individuals, individuals with nonmalignant diseases, and individuals with malignant diseases are presented.

MATERIALS AND METHODS

Purification of MAD-2. High concentrations of MAD-2 have been previously described to be present in tumor ascitic fluid from patients with ovarian carcinoma (12). Ascitic fluid was used as a source of MAD-2 for all purification procedures, due to the accessibility of large volumes of this material. Immediately after collection, fluid samples were cooled to 4 °C, and PMSF was added to a final concentration of 0.5 mM. The samples were centrifuged at 1000 × g for 20 min, and the supernatants were frozen at −20 °C in ~400-ml aliquots. All steps of the purification procedure were conducted at 4 °C.

For each purification an aliquot of fluid was thawed, and PMSF was again added to a concentration of 0.1 mM fresh material. The sample was dialyzed overnight in 8 liters of 50 mM potassium phosphate buffer (pH 6.8), containing 1 mM EDTA and 0.1 mM PMSF. The dialyzed sample was applied directly to a 5- × 36-cm DNA-cellulose column (~1200 mg DNA) which had been equilibrated in Buffer A [10 mM potassium phosphate buffer-1 mM EDTA-0.1 mM PMSF (pH 6.8)] containing 50 mM NaCl at ~5 ml/min. The column was successively washed with 600-ml volumes of Buffer A, containing 50 mM NaCl, 150 mM NaCl, and then 1 M NaCl. The eluate was collected in 15-ml fractions, and protein was monitored by the optical density at 280 nm. The fractions containing the peak of protein which eluted in the 1 M NaCl wash were pooled, and PMSF was added to a concentration of 0.1 mM fresh material. This sample was dialyzed in 10 volumes of 10 mM Tris-HCl buffer [1 mM EDTA-0.1 mM PMSF (pH 7.8)] and then applied to a 10-ml column of gelatin-Sepharose (~10 mg gelatin per ml Sepharose) at 60 ml/hr. The gelatin-Sepharose column was washed with 50 ml Buffer B [10 mM Tris-HCl buffer-150 mM KCl-1 mM EDTA-0.1 mM PMSF (pH 7.8)], 50 ml Buffer B and 1 M urea, and MAD-2 was next eluted with 50 ml of Buffer B containing 4 M urea, at the same flow rate at which sample application was performed. The protein peak which eluted with 4 M urea was pooled and dialyzed in Buffer B.

Protein in this fraction was reduced with 10% sodium dodecyl sulfate. Reduced proteins were analyzed by sodium dodecyl sulfate gel electrophoresis into two major protein fractions (1,7,15). This approach has involved the comparison of DNA-binding protein fractions from normal sera to analogous fractions from sera of individuals with malignant disease, attempting to visualize unique species in the sera of cancer patients. Two human serum DNA-binding proteins have been observed to be present at elevated levels in the sera of individuals with malignant disease (7, 12). The first detected malignant disease-associated serum DNA-binding protein, termed C3DP, was isolated and identified as a fragment of complement component C3 (13). An assay system was developed for C3DP, and preliminary clinical studies have indicated its usefulness as a tumor marker (14, 16). A second protein, designated MAD-2, was recently reported to be present in a fraction eluted with 0.6 M NaCl from DNA-cellulose (12) and was shown to be distinct from C3DP. Elevated MAD-2 levels have been reported to be present in the sera from a high percentage (69%) of individuals with carcinomas of various primary sites.

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affinity chromatography on gelatin-Sepharose, as described by Engvall et al. (5).

MAD-2 Antiserum Production. Rabbits were inoculated s.c. with 60 μg MAD-2 in complete Freund's adjuvant, followed by a second injection of 60 μg in complete Freund's adjuvant 1 month later. Antiserum were obtained from blood collected at bleedings on the seventh and ninth weeks following the initial injection.

Radioimmunoassay for MAD-2. The radioimmunoassay for MAD-2 is essentially the same as that previously described for C3DP (16). MAD-2 was iodinated by the solid-phase lactoperoxidase technique (4) to specific activities of 10 to 20 μCi 125I per μg protein. The reaction mixtures of antisera, 125I-MAD-2, and the MAD-2 sample were incubated at room temperature in Falcon Microtest II, microtiter plates (Falcon Plastics, Oxnard, Calif.) for at least 12 hr prior to the addition of Sepharose-coupled goat (anti-rabbit IgG) IgG beads. The plates were gently mixed on a rotary shaker at room temperature for 6 hr, and then the Sepharose beads were collected on Whatman GF/A glass fiber filters (Whatman, Inc., Clifton, N. J.), using a Brandel Model M24V cell harvester (Rockville, Md.).

Affinity Chromatography Materials. DNA-cellulose was prepared from calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.), as described by Litman (9). Gelatin (Knox Gelatine, Inc., Johnstown, N. Y.) was coupled to Sepharose 4B at a concentration of 10 mg/ml using the method of Cuatrecasas et al. (3).

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (8), and the gels were stained with Coomassie blue as described by Fairbanks et al. (6).

Protein Determination. Protein was quantitated by the method of Lowry et al. (10), using bovine serum albumin as a standard.

RESULTS

Isolation of MAD-2. Previous studies (12) detected a protein species, termed MAD-2, at elevated concentrations in the sera of patients with neoplastic diseases. The original assay system developed for this protein did not permit precise quantitative determinations of MAD-2 levels in serum samples. Studies were initiated to purify MAD-2 and develop an antiserum against it for the development of a quantitative radioimmunoassay system.

MAD-2 was purified approximately 500-fold using a chromatographic procedure involving fractionations with DNA-cellulose and gelatin-Sepharose (Fig. 1) as described in "Materials and Methods." Human effusion fluids, obtained either from cancer patients with metastatic disease involving the pleura or ovarian cancer patients with malignant ascites, were used as sources for purification of MAD-2. Strict precautions were taken to limit proteolysis during all purification steps by including a serine-protease inhibitor (PMSF) and a metal-chelating agent (EDTA) in buffers throughout the purification procedure. MAD-2 purified from malignant effusion fluids migrated as 2 major protein bands with molecular weights of approximately 200,000 and 210,000, which appeared to be similar to the MAD-2 bands observed in the gel patterns from serum samples. An antiserum directed against MAD-2 purified in this manner was produced in rabbits as described in "Materials and Methods."

Identification of MAD-2. Purified MAD-2 was found to react with an antiserum directed against human Clg, kindly supplied by Dr. M. Mosesson (State University of New York, Brooklyn, N. Y.), as shown in Fig. 2. The smooth intersection of precipitin arcs between purified MAD-2 (Well D) and antiseras directed against either human Clg (Well B) or purified MAD-2 (Well A) indicated a high degree of immunological identity between Clg and MAD-2. This antigenic similarity was further indicated by the smooth precipitin arcs between an antiserum directed against human Clg (Well B) and either purified MAD-2 (Well D) or human plasma (Well E).

The radioimmunoassay was used to quantitatively analyze the immunoreactivity of these 2 protein species to further demonstrate that both MAD-2 and Clg share antigenic determinants. Purified preparations of either MAD-2 or Clg were used to competitively inhibit the binding of 125I-MAD-2 by an antiserum directed against MAD-2 as shown in Chart 1. Both protein preparations revealed similar inhibition curves, demonstrating that the cross-reactivity between MAD-2 and Clg was not simply due to trace amounts of Clg present in MAD-2 preparations.

Purified MAD-2 and Clg were compared using SDS-gel electrophoresis (Fig. 3). As previously reported (11), Clg was found to be composed of 2 polypeptide chains with molecular weights of about 220,000, which are disulfide bonded together. MAD-2 polypeptides migrated corresponding to smaller chains with weights of 200,000 to 210,000, which did not exist as higher molecular weight covalently bonded complexes. These studies suggested that MAD-2 may be a large fragment of either Clg or its immunologically identical analog, cellular fibronectin, which has been generated as the result of proteolytic degradation.

MAD-2 Quantitation. Studies were initiated to determine the correlation between quantitative MAD-2 values determined with the radioimmunoassay system and the SDS-gel electrophoresis-banding intensities. Radioimmunoassays were performed on 92 samples of DNA-binding protein fractions, which had been obtained in the previous study (12), for the determination of the relative MAD-2-banding intensities. The results of these assays were calculated to reflect the amount of MAD-2 present.
Analyses of the data from these quantitative MAD-2 determinations, with respect to the disease category of the patients from which the sera were obtained (Chart 3), indicated that 61% of the samples from the malignant disease category had values of 20 \( \mu g/ml \) or greater, whereas only 8% of the samples from the normal and nonmalignant disease category had values greater than 20 \( \mu g/ml \). The panel of samples analyzed in this study was obtained from the series which was assayed in the previous report (12). The normal and nonmalignant disease category was composed primarily of patients with cirrhosis, pulmonary disease, and pancreatitis, along with normal individuals. The malignant disease samples were obtained from individuals with carcinomas from various organ sites (ovary, breast, pancreas, and colon). Correlations between disease status and marker levels were not made in this study. These results offer preliminary confirmation of the initial observations (12) that elevated levels of MAD-2 are detectable in the sera from individuals with malignant diseases.

**DISCUSSION**

This report describes the isolation and identification of the human serum DNA-binding protein, MAD-2, which has been previously shown to be present at elevated concentrations in cancer patients (12). Purified MAD-2 is immunologically identical to human Clg, and it has been resolved into a group of 2 major protein bands with molecular weights of 200,000 and 210,000 by SDS-polyacrylamide gel electrophoresis. These protein species are not disulfide bonded; however, gel filtration studies suggest that they exist as noncovalently bonded dimers. MAD-2 polypeptides appear smaller than the chains of Clg with molecular weights of 220,000. The molecular weight heterogeneity in MAD-2 preparations may be the result of proteolytic degradation and possibly of altered glycosylation patterns which affect the electrophoretic mobility. MAD-2 retains the capacity to bind collagen (as used in the purification procedure), which is characteristic of Clg and cellular fibronectin. These data suggest that MAD-2 represents the major portion of either the Clg or cellular fibronectin molecules minus the carboxy-terminal segment which contains the disulfide bond linking the 2 subunits (18).

Previous studies by Chen et al. (2) have indicated the presence of multiple molecular forms (proposed degradation products) of Clg in plasma, and have further shown that proteases such as plasmin are capable of generating fragments from Clg with molecular weights similar to those of purified MAD-2. These authors did not measure changes in the amount of Clg fragments in different diseases; however, they may have been detecting species similar to those seen in this study. MAD-2 may be generated by either tumor-associated proteases or possibly by proteases released by lymphocytes during an immune response directed against the tumor. A variety of studies have identified different protein degradation products which appear to be associated with malignant diseases [recently reviewed by Ruddon (17)], including the other cancer-associated DNA-binding protein C3DP. The quantitation of these degradation products in biological fluids appears to be an important tool for monitoring tumor growth activity in cancer patients (14).

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R. G. Parsons, unpublished results.

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Chart 2. Correlation between MAD-2 band intensities detected by SDS-polyacrylamide gel electrophoretic analysis and immunological MAD-2 quantitation on DNA-binding proteins obtained from human serum samples as previously described (12).

Chart 3. MAD-2 levels in DNA-binding protein fractions from sera of either normal individuals and patients with nonmalignant diseases or patients with malignant diseases. DNA-binding protein fractions were obtained from serum samples as previously described (12).
The preliminary data presented in these studies support and confirm previous observations (12) that MAD-2 is present at elevated levels in the sera of patients with malignant diseases. The detection of elevated values (>20 µg/ml) in 61% of the sera from patients with malignant diseases is consistent with the 69% elevated levels which were previously determined (12) on a larger group of serum samples, but by a less quantitative assay. Currently, a more sensitive serum assay system for MAD-2 is being developed similar to the C3DP assay (14), which will permit the quantitative determination of MAD-2 levels in 10- to 25-µl serum samples to facilitate the rapid analysis of a greater number of samples. This will allow longitudinal studies of cancer patients during therapy programs to determine the prognostic value of this marker for the therapeutic maintenance of patients with malignant diseases. Further clinical studies will involve comparison of MAD-2 and C3DP to determine whether the 2 proteins are generated concordantly or whether the analysis of both markers together will increase the diagnostic and prognostic usefulness of either marker alone.

REFERENCES

Fig. 1. SDS-polyacrylamide gel electrophoretic profiles of fractions from the MAD-2 purification procedure. The gel was composed of 7.5% polyacrylamide, and the samples were reduced with 1% 2-mercaptoethanol prior to electrophoresis. A, whole peritoneal fluid (ovarian carcinoma); B, 1 M NaCl DNA-cellulose eluate; and C, 4 M urea gelatin-Sepharose eluate.

Fig. 2. Immunodiffusion studies of purified MAD-2. A and C, rabbit antisera directed against purified MAD-2; B, rabbit antisera directed against purified human Clg; D and F, purified MAD-2; and E, human plasma.

Fig. 3. Gel electrophoretic profiles of purified MAD-2 and Clg on 5% polyacrylamide gels in the presence of 0.1% SDS. R, reduced and alkylated samples; NR, nonreduced protein samples. Protein standards which were used for determining the relationship between mobility and molecular weight were phosphorylase A, β-galactosidase, human complement component C3, myosin, and human Clg.
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