IDENTIFICATION OF THE SPECIES-SPECIFIC ANTIGENIC DETERMINANT(S) OF HUMAN PLASMA FIBRONECTIN BY MONOCLONAL ANTIBODIES

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1. Introduction

Fibronectin is a high-M_r glycoprotein which is found in the blood plasma and other body fluids, in connective tissues and in basement membrane [1–7]. Fibronectin is involved in a variety of cell functions – adhesion on different surfaces, cell motility, phagocytosis [1–7]. Fibronectin is a multidomain disulfide-bonded dimer, the dimensions of the molecule are 15 × 8 nm; β-form is the main element of secondary structure [8,9]. Fibronectin has an affinity to the cell surface, collagen, heparin, fibrinogen, DNA, actin and some other macromolecules [1–7]. Similar chemical properties and functions are characteristic for fibronectins isolated from different sources. Moreover, conventional antibodies generated against one of the types of this protein usually exhibit significant cross reactivity with fibronectins of the other origin [10,11]. The immunologic similarity of fibronectins can be illustrated by the fact that antibodies against human plasma fibronectin cross-react with the homologous protein from sponges [12]. However, monoclonal antibodies have been obtained which distinguish between different forms of this protein [13–15], thus demonstrating the difference between plasma and cellular fibronectin [13]. In [14,15] monoclonal antibodies specifically recognizing fibronectin from amniotic fluid were described. Monoclonal antibody recognizing a single antigenic determinant can provide a sharp experimental tool for the conformational and functional analysis of large multidomain proteins. For example, monoclonal antibodies were very useful in the localization and isolation of the cell adhesion fragment and the site of structural difference between cellular and plasma forms of fibronectin [13,14].

We describe here species-specific monoclonal antibodies sensitive to the conformational transitions of the fibronectin molecule. We have also localized the antibody-binding site, i.e., the antigenic determinant specific for human and rhesus macaque plasma fibronectin.

2. Materials and methods

Fibronectin was isolated from human plasma by affinity chromatography on gelatin–Sepharose followed by ion-exchange chromatography on Whatman DE-52 cellulose [16]. The characteristics of our human plasma fibronectin preparation have been published [9]. Fibronectins from pig, rat, calf, chicken and rhesus macaque plasma were isolated by affinity chromatography on gelatin–Sepharose and concentrated by ammonium sulfate precipitation (40% saturation).

Hybridomas secreting antibodies specific for fibronectin were prepared by the general method in [17]. BALB/c mice were immunized with intravenous injections of fibronectin (100 μg), followed by booster injections of the same amount of protein after 45 days. After 4 days the spleens were excised, and the spleen cells were fused with mouse myeloma cells P3/NSI-1-Ag 4-1. After growth on a selective medium containing hypoxanthine, aminopterin and thymidine the hybrid cells were seeded into 24-well plates (Lindbro, Flow Labs). Positive clones secreting antifibronectin antibodies were detected by a solid-phase radioimmunoassay; these were then subcloned by limiting dilution and expanded as ascites tumors in BALB/c mice. The antibodies were precipitated from

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ascites fluid with sodium sulfate (18% saturation), dissolved in the 0.2 M Na_3BO_3, 0.15 M NaCl (pH 8.0) and dialyzed against the same buffer. The clone used in this work was marked 2F. Antibodies synthesized by this cell line belong to the class IgG1, κ. Conventional antifibronectin antibodies were affinity-purified in fibronectin–Sepharose.

Fibronectin–antibody interaction was tested by enzyme-linked immunoassay (ELISA) [18]. Polysterene 96-well microtiter plates (Falcon) were incubated overnight at 4°C with a solution of fibronectin in sodium carbonate buffer. To test the effect of heat denaturation on fibronectin–antibody interaction, fibronectin was heated for 5 min prior to coating the plate and immediately cooled to 4°C. The unbound antigen was removed by washing with phosphate-buffered saline, containing 0.05% Tween 20. Monoclonal or conventional antifibronectin antibodies were allowed to interact with fibronectin for 1 h at 20°C. Goat (anti-rabbit IgG) IgG and rabbit (anti-mouse IgG) IgG containing conjugated horseradish peroxidase were used as a second layer for detecting conventional and monoclonal antibodies, respectively.

To isolate short fibronectin fragments, the protein (3 mg/ml) was cleaved by trypsin (30 μg/ml) in 0.02 M Tris–HCl (pH 7.4), 4 M urea at 20°C for 6 h [19]. Trypsin was added in two portions with a 3-h interval [19]. The electrophoretic analysis did not reveal the peptides of $M_r > 30\,000$. Monoclonal antibody-binding fragments were isolated by affinity chromatography on monoclonal antibody–Sepharose (1 mg IgG/ml gel).

3. Results and discussion

Fibronectin is a strong antigen and antifibronectin antibodies are known to exhibit the significant cross reactivity with fibronectins from different sources [10,11]. The data shown in fig.1 demonstrate the ability of conventional anti-human plasma fibronectin antibodies to interact with fibronectins from different biological species. These antibodies practically do not distinguish between human, pig, rat and rhesus macaque plasma fibronectins, while their affinity to the homologous protein from calf and chicken plasma though somewhat lower, is still high. Thus, we have obtained monoclonal antibodies against human plasma fibronectin which exhibit a certain species specificity. These antibodies recognize fibronectins from human and rhesus macaque plasma, but do not interact with

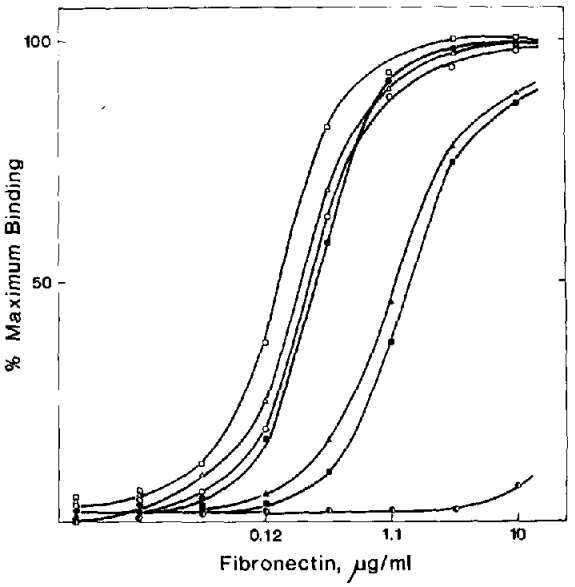

Fig.1. Interaction of conventional antibodies against human plasma fibronectin with fibronectins, obtained from plasma of different species; 100% = the plateau level in the reaction of antibodies with human-plasma fibronectin; antibodies were 3 μg/ml; (□) human fibronectin; (△) pig fibronectin; (○) rat fibronectin; (●) rhesus macaque fibronectin; (▲) calf fibronectin; (■) chicken fibronectin; (◐) non-immune rabbit IgG instead of antifibronectin antibodies.

rat, calf, pig and chicken plasma fibronectins (fig.2). Thus, in spite of the chemical similarity of fibronectins from different biological species, monoclonal antibodies reveal the antigenic determinant which is shared by human and rhesus macaque plasma fibronectins, but is absent in the homologous proteins from rat, pig, calf and chicken plasma.

To isolate short monoclonal antibody-binding fragments of fibronectin, trypsin digestion in the presence of 4 M urea was carried out. Cleavage products, containing peptides with $M_r < 30\,000$ were adsorbed on monoclonal antibody–Sepharose. The retained fraction was eluted and analyzed. The absorption was shown to be specific as the fragments were able to interact with monoclonal antibody again (fig.2A). Eluted fragments were found to be of M_r 25 000–27 000 by SDS–polyacrylamide gel electrophoresis (fig.2). Thus, the antigenic determinant which is recognized by monoclonal antibody in this investigation does not exceed 10% of the fibronectin monomer polypeptide chain. The fibronectin molecule contains several structural domains which are resistant to proteolytic cleavage [8,9,20,21]. To test whether mono-

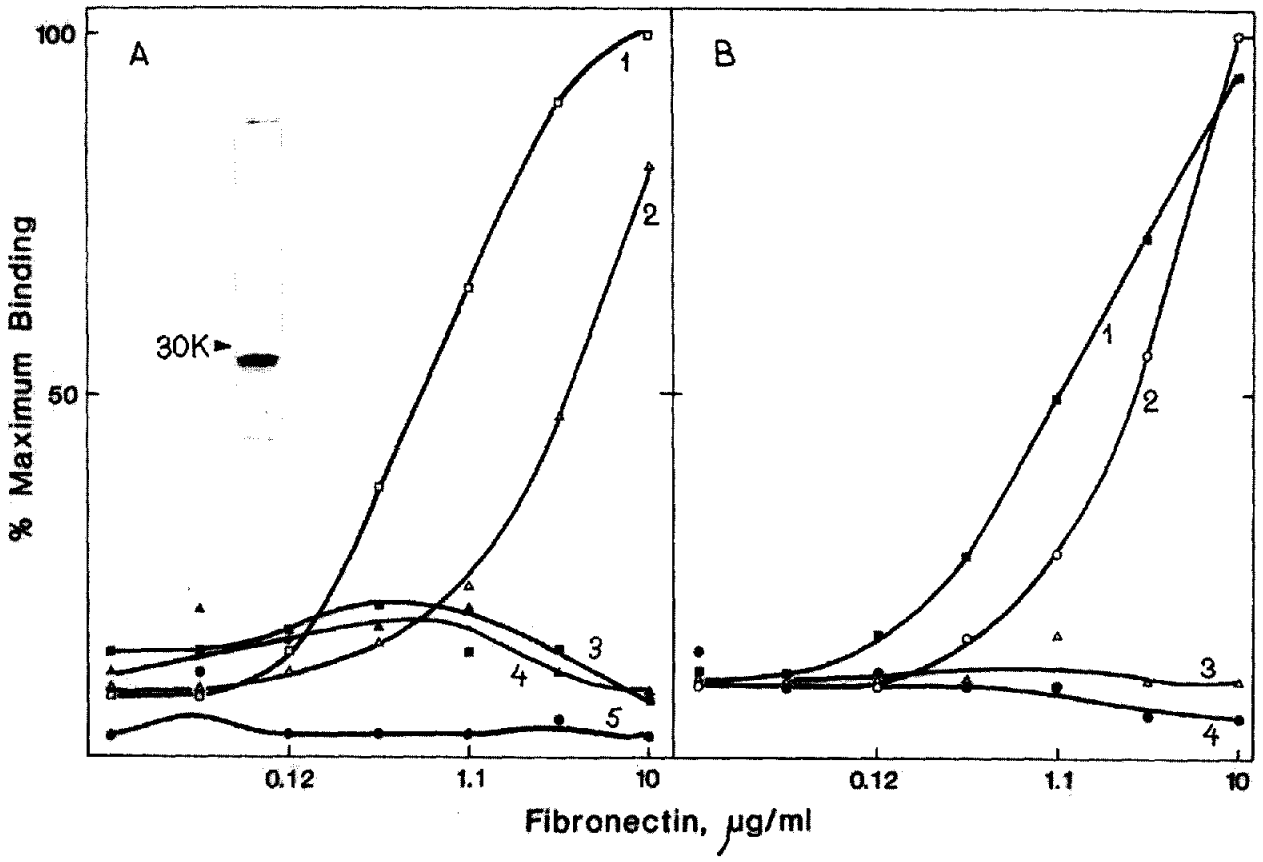

Fig.2. Interaction of monoclonal antibodies against human-plasma fibronectin with fibronectins obtained from plasma of different species; 100% = the plateau level in the reaction of antibodies with human plasma fibronectin; antibodies were 12 μg/ml; (A) 1, human fibronectin; 2, the tryptic fragments retained on monoclonal antibodies–Sepharose (electrophoregram in the left upper corner); 3, calf fibronectin; 4, chicken fibronectin; 5, instead of monoclonal antibodies non-immune mouse IgG were added; (B) 1, human fibronectin; 2, rhesus macaque fibronectin; 3, rat fibronectin; 4, pig fibronectin.

clonal antibody-binding site is a part of the structural domains we studied the effect of heat and pH-denaturation on the ability of fibronectin to interact with monoclonal antibodies. The antigenic determinant was not disturbed by heating of the protein to 60°C or by a weak alkaline pH (fig.3). Heating to 70°C and 80°C causes the loss of the affinity of monoclonal antibodies to denatured fibronectin. These data are in good agreement with the results of spectroscopic, calorimetric, electron microscopic and sedimentational studies of fibronectin, as well as with conformational analysis of the protein with conventional antibodies [8,9,22]. In [9] heat denaturation of the fibronectin molecule occurred in several steps and began at ~60°C

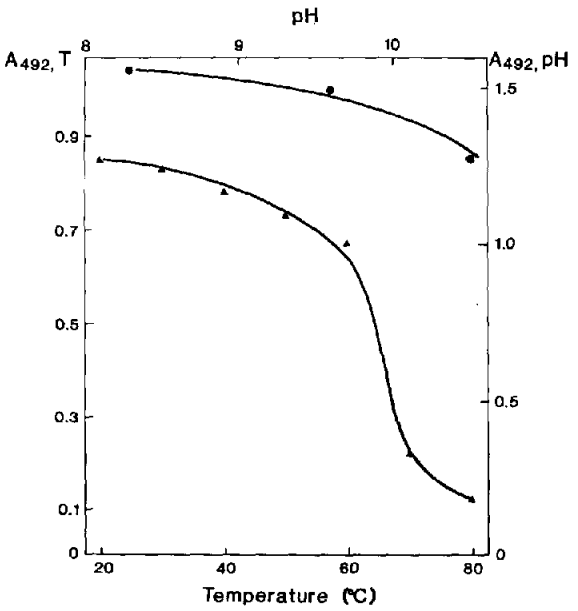

Fig.3. Effect of heating and alkaline pH on monoclonal antibodies–fibronectin interaction; antibodies were 0.2 μg/ml; (▲) heat denaturation; (●) alkaline pH denaturation.

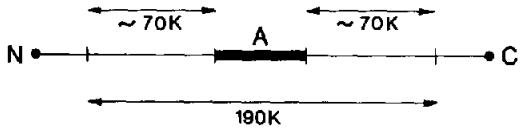

Fig.4. Model of the fibronectin monomer showing the position of biologically active tryptic fragments according to [21]; (▬) monoclonal antibody-binding site (A); K, $M_r \times 10^{-3}$.

[9]. However, at alkaline pH the protein molecule undergoes unfolding which is not accompanied by the disruption of the domains [8]. Thus, the monoclonal antibody-binding fragment of the fibronectin molecule seems to be a structural domain or at least a part of one.

To define the position of our monoclonal antibody-binding fragment on the fibronectin molecule we obtained a tryptic 200 000–190 000 M_r fragment that contains both heparin- and gelatin-binding sites, 70 000 M_r gelatin- and 70 000 M_r heparin-binding fragments. These fragments are known to be localized close to two terminals of the molecule (fig.4). The 70 000 M_r gelatin- and heparin-binding fragments do not interact with the monoclonal antibody. However, a large 200 000–190 000 M_r fragment, that contains both gelatin- and heparin-binding sites, interacts with our monoclonal antibody. These results suggest that the monoclonal antibody-binding site is located in the central part of the polypeptide chain (fig.4).

Thus, anti-human plasma fibronectin monoclonal antibodies, possessing a certain species specificity, interact with a 25 000–27 000 M_r structural domain of the fibronectin moleeule, located in the central part of the protein polypeptide chain.

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