MONOCLONAL ANTIBODIES DIRECTED TO TWO DIFFERENT DOMAINS OF HUMAN PLASMA FIBRONECTIN: THEIR SPECIFICITIES

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1. Introduction

Fibronectin is a class of adhesive glycoproteins present at the cell surface, intercellular matrices, plasma and other body fluids, sharing properties such as:

(1) A high M_r (>400 000) consisting of 2 disulfide-linked subunits α and β, though these subunits may or may not be identical;
(2) Binding to collagen, heparin and fibrin, and inducing cell adhesion and spreading (reviews [1–4]).

The functional domains having these binding activities have been isolated and characterized [5,6]. The deletion of fibronectin from the cell surface, associated with oncogenic transformation [7,8], may be related to a decreased adhesiveness and increased invasive properties of cancer cells [9,10]. Polyclonal antibodies directed to fibronectin are cross-reactive irrespective of their sources and species [1–4] and have been used as a reagent to detect and quantify fibronectin or its related compounds. We now report two monoclonal antibodies directed respectively to the collagen-binding domain and to the domain which binds to heparin and induces cell adhesion and spreading. While this work was in progress, papers describing monoclonal antibodies directed to fibronectin have been published [11–14], although the specificities of antibodies described are entirely different from those reported here.

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Abbreviation: PBS, phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.4)

2. Materials and methods

2.1. *Purification of fibronectin and its proteolytic fragments*

Plasma fibronectins of various species were purified by affinity chromatography on gelatin–Sepharose as in [5,6]. Cellular fibronectin purified from conditioned medium of WI-38 human fibroblasts was kindly provided by Dr W. G. Carter. Cell-surface fibronectin of chick embryo fibroblasts was isolated as in [15]. The domain fragments of human plasma fibronectin were isolated after thermolysin digestion as in [6].

2.2. *Production of hybridomas*

Hybridomas producing monoclonal antibodies were prepared as in [16]. Balb/c mice were immunized with human plasma fibronectin in complete Freund's adjuvant. The antigen solution contained 3.5 mg fibronectin/ml, and 75–100 μl were injected/mouse. Four subcutaneous injections were performed at 10-day intervals. Cell fusion with the mouse myeloma NS/1 was performed 3 days after the last immunization. Hybrids producing antibodies were screened and minicloned in microtiter wells as in [17,18]. Two hybridoma clones were isolated; these are called D5 and H5, respectively. Immunoglobulin class of these antibodies was determined with rabbit antisera specific for various mouse immunoglobulin classes (Litton Bionetics, Kensington, MD 20795) according to [19].

2.3. *Determination of the antibody specificity*

The affinity of antibodies to fibronectins from different species was tested by solid-phase competitive radioimmunoassay as in fig.1. The blocking activity of the antibodies for gelatin-binding and cell attach-

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ment mediated by fibronectin has been tested as follows: Plastic plates (Dynatech/'Microelisa plates') were first coated with fibronectin by incubating for 2 h with a solution of fibronectin in PBS containing 10 μg/ml. The plates were then blocked by incubating for 2 h with 5% bovine serum albumin in PBS. The plates were washed 3 times with PBS and incubated with the antibodies for 45 min, followed by the addition of the ^{125}I-labeled gelatin or NIL cells metabolically labeled with [^{3}H]leucine. The inhibitory effect of antibodies can be observed by the decrease of radioactivity bound to the fibronectin coat. The reactivity of fibronectin fragments to antibodies was tested on polyacrylamide gel electrophoresis followed by transferring onto a nitrocellulose sheet and reacting with antibodies [20].

3. Results

3.1. *Two hybridoma lines, producing IgG$_1$ antibodies, specific for human fibronectin*

Two hybridoma cell lines, D5 and H5, were established and characterized to produce IgG$_1$ antibodies. These antibodies seem to be specific to human fibronectin, because they reacted strongly with both human plasma and cellular fibronectin but not with fibronectin from bovine, guinea pig, mouse or chicken (fig.1).

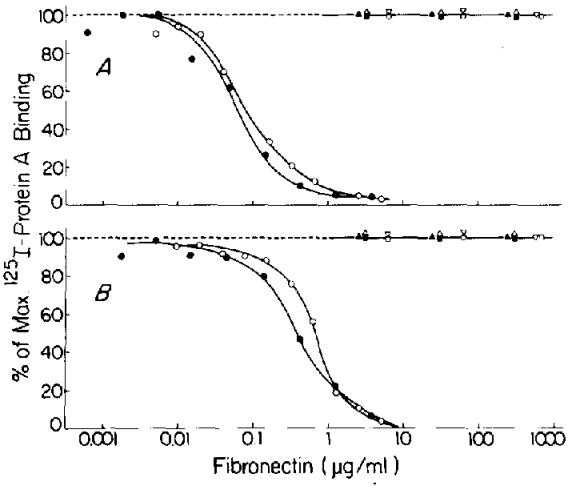

Fig.1. The affinities of H5 (A) and D5 (B) antibodies for fibronectins of various species. Polystyrene microtiter plates were coated with either 1 μg/ml (for H5) or 0.1 μg/ml (for D5) of human fibronectin and then incubated with 5% bovine serum albumin. The plates were washed with PBS then incubated with antibodies to which were added serially-diluted fibronectins from different species. The plates were washed and then incubated sequentially with rabbit anti-mouse immunoglobulin and ^{125}I-labeled protein A: (○) human plasma fibronectin; (●) human cellular fibronectin; (△) fibronectin from fetal bovine serum; (▲) fibronectin from newborn bovine serum; (□) guinea pig plasma fibronectin; (■) chicken cellular fibronectin; (▽) mouse plasma fibronectin.

3.2. *Domain specificity of two hybridoma antibodies*

The specificity of two hybridoma antibodies in their reactivity to different domains isolated after proteolysis was determined first by plate assay [17,18]. The antibody produced by the hybridoma D5 reacted specifically with the gelatin-binding domain with 40 000 M_r. In contrast, the antibody produced by the hybridoma H5 reacted specifically with the 150 000–140 000 M_r fragment which represents the domain capable of binding to heparin and inducing cell attachment and spreading. These results were further confirmed by gel electrophoresis of proteolytic digests of fibronectin followed by transferring onto nitrocellulose membrane and reacting with antibodies. Among 4 different domain fragments produced by thermolysin digestion, H5 reacted with only 150 000–140 000 M_r

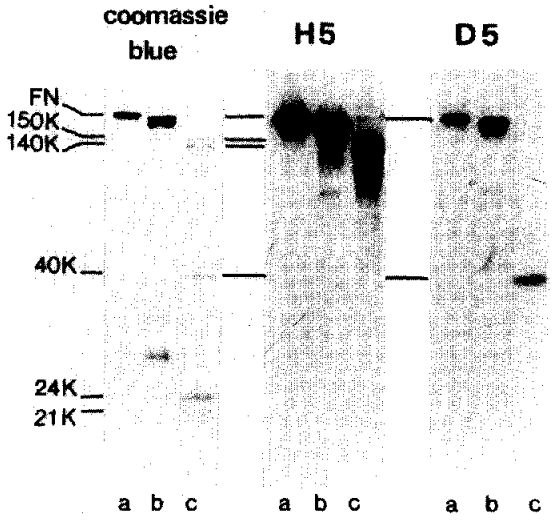

Fig.2. Domain specificity of H5 and D5 monoclonal antibodies. Human plasma fibronectin was digested at room temperature by either trypsin (1 μg/ml, 30 min) or thermolysin (5 μg/ml, 4 h). The digests were analyzed on SDS–polyacrylamide gel electrophoresis in triplicate. One group was stained by Coomassie blue (left). The other two groups were blotted on nitrocellulose and incubated with H5 (middle) and D5 (right), followed by sequential incubation with rabbit anti-mouse immunoglobulin and ^{125}I-labeled protein A: (a) intact fibronectin; (b) trypsin digest; (c) thermolysin digest.

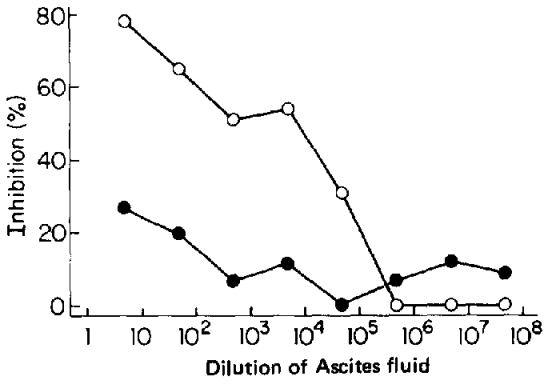

Fig.3. Inhibition of the binding of ^{125}I-labeled gelatin on fibronectin-coated plate. Fibronectin-coated plate was incubated with H5 (•) or D5 (○) ascites in serial dilutions, then incubated with ^{125}I-labeled gelatin.

fragments, whereas D5 only reacted with 40 000 M_r fragment (fig.2c). Similarly, tryptic 200 000 and 180 000 M_r fragments which contain both 150 000–140 000 and 40 000 M_r domains were also heavily stained by both H5 and D5 (fig.2b).

3.3. *Antibody blocking of gelatin-binding and cell attachment*

The antibody specific to gelatin-binding domain (D5) was found to block the binding of radioactive gelatin on the fibronectin-coated plate, whereas the antibody directed to cell-binding domain (H5) did not block the binding of radioactive gelatin on the fibronectin layer (fig.3).

The effect of monoclonal antibodies on the fibronectin-mediated cell attachment was studied on fibronectin-coated plates with or without the addition of the monoclonal antibodies (table 1). There was no preferential inhibition of cell attachment by either antibody directed to 150 000–140 000 M_r or 40 000 M_r domain.

Table 1
Effect of monoclonal antibodies on the FN-mediated cell attachment

Substrate	Antibody (ascites[a])	Cell attachment (cpm)
Human FN	None	4.174 (100%)
Human FN	Anti-150 000–140 000 M_r (H5)	3.606 (86%)
Human FN	Anti-40 000 M_r (D5)	3.610 (86%)
Human FN	Anti-mouse H-2D	3.932 (94%)
BSA	None	0.171 (4%)
Hamster FN	None	4.022 (96%)

[a] Dilution of ascites: ×10

4. Discussion

Because of their narrow specificities, monoclonal antibodies can be used as probes to pinpoint a specific region of a protein. Since fibronectin has been shown to consist of at least 4 distinct functional domains [6], monoclonal antibody specific to each domain is a powerful tool for the detailed analysis of its structure–function relationship. We produced two such antibodies against human plasma fibronectin with different domain specificities. One of them, D5, is directed to 40 000 M_r gelatin-binding domain and the other, H5, is directed to 150 000–140 000 M_r domain. D5 inhibits the interaction of gelatin with fibronectin. Interestingly, H5 did not interfere with fibronectin-mediated cell attachment although polyclonal anti-fibronectin antibodies generally inhibit fibronectin-mediated cell attachment [21]. In [14], a monoclonal antibody directed to the small locus of cell adhesion site of fibronectin blocked cell adhesion and spreading. The small locus represents a polypeptide region of 15 000 M_r that promotes cell adhesion and spreading. Therefore, H5 does not recognize the same locus or domain as recognized by the monoclonal antibody in [14].

The majority of polyclonal antibodies prepared for any species of fibronectin are cross-reacted to fibronectins of different species although species-specific antibodies can be prepared by absorbing antibodies with fibronectins of certain species [22]. The antibodies reported here may be useful, because of their narrow species specificity, to study the distribution of human fibronectin as injected in different species. The observation that the antibody directed to 40 000 M_r domain (D5) did not inhibit cell attachment but inhibited binding of gelatin to fibronectin clearly indicates that cell attachment on fibronectin is different from the gelatin-binding property and is not mediated through this domain.

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