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TWO NOVEL MONOCLONAL ANTIBODIES TO FIBRONECTIN THAT RECOGNIZE THE HEP II AND CS-1 REGIONS RESPECTIVELY: THEIR DIFFERENTIAL EFFECT ON LYMPHOCYTE ADHESION

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SUMMARY: We have obtained two new mAbs to the carboxy-terminal region of fibronectin, namely P3D4 and P1F11, and have studied their binding sites and their ability to block lymphocyte adhesion to fibronectin. ELISA and Western blot analyses showed that P3D4 reacts with both fibronectin chains and both Hep II-containing fragments (58 kDa and 38 kDa). P1F11, raised against the synthetic peptide CS-1, reacted with the 38 kDa fragment and with a 190 kDa fragment derived from the A chain of fibronectin. P1F11 did not react with the 58 kDa fragment thus clearly establishing that 58 kDa comes from the B chain of fibronectin and lacks the CS-1 sequence. mAbs P3D4 and P1F11 were used to evaluate the contribution of the Hep II and CS-1 sites in cell attachment to fibronectin. P3D4 effectively inhibited B cell adhesion to 38 kDa, 58 kDa and fibronectin; P1F11 however produced only limited inhibition, suggesting that lymphocyte interaction with Hep II may modulate further binding to the CS-1 site.

We (1-3) and others (4) have previously shown that lymphocyte interaction with fibronectin (Fn) involves multiple sites located in the central and carboxy-terminal regions of Fn. These sites can be isolated within tryptic fragments of 80 kDa (RGD sequence), 58 kDa and 38 kDa (Hep II domain), which promote cell adhesion efficiently (1-3). The 38 kDa fragment also contains the CS-1 adhesive sequence (5, 6) of the alternatively spliced IIICS region of Fn, and is derived from the A chain of Fn. Based on enzyme specificity and on the molecular weight of the resulting Fn fragments, it has been assumed that the 58 kDa fragment is derived from the B chain of Fn (1-3). However the origen of this fragment has not been clearly established yet.

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Abbreviations: Fn, fibronectin; mAbs, monoclonal antibodies.

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The existence of two adhesive sites (Hep II and CS-1) in close proximity within Fn, may suggest a functional cooperation between both sites that results in an efficient cell attachment. Using a panel of monoclonal antibodies (mAbs) selected for their ability to inhibit lymphocyte adhesion to Fn, we have identified the $\alpha 4\beta 1$ integrin as the receptor for both sites, Hep II and CS-1 (1, 3). In the present study we have obtained two novel mAbs to Fn, which specifically recognize these adhesive sites. We have used these mAbs to clearly establish that the 58 kDa fragment lacks CS-1 and is derived from the B chain of Fn. We have also studied the contribution of both sites (Hep II and CS-1) to cell attachment by testing the effect of these mAbs on lymphocyte adhesion to Fn and its fragments.

MATERIALS AND METHODS

Fibronectin and fibronectin fragments. Human plasma Fn was the generous gift of Drs. B. Horowitz and R. Shulman (New York Blood Center, New York, NY). Tryptic fragments of 200 and 190 kDa representing the B and A chains of Fn respectively, minus the 29 kDa N-terminal and the 6 kDa C-terminal domains were obtained as described (6). Tryptic fragments of 38 kDa, 58 kDa, and 80 kDa were produced as previously reported (1-3, 6). Briefly, these fragments are isolated by heparin-Sepharose affinity chromatography of the Fn digests and eluted in the 0.1 M NaCl fraction (80 kDa), or 0.5 M NaCl fraction (38 and 58 kDa) from this column. The 38 and 58 kDa fragments are further resolved by DEAE-Sephacel, which retains the 58 kDa but not the 38 kDa fragment. Further purification of the 38 kDa fragment was achieved by a CM-Sephadex matrix equilibrated in 10 mM Tris, 2 M urea, 50 mM NaCl pH 7.0. After removal of unbound materials with this buffer, the 38 kDa fragment was separated from other contaminants by running a NaCl gradient (50-300 mM) through this column. Fragments were finally dialyzed against PBS, concentrated and stored in aliquots at -70° C.

Antibodies. mAbs to Fn were produced by immunizing RBF/DnJ mice with the previously described synthetic peptide CS-1 (DELPQLVTLPHPNLHGPEILDVPST) (5) coupled to keyhole limpet hemacyanin (KLH), or with the 38 kDa Fn fragment, using previously reported protocols (1). Polyclonal antibodies to the 38 kDa fragment were obtained by subcutaneous injection of New Zealand White rabbits with the purifed fragment.

Cell attachment assays. The human B cell line Ramos was obtained from the American Type Culture Collection (Rockville, MD) and expanded in RPMI, 10% heat-inactivated fetal bovine serum (Gibco Life Technologies, Middlesex, UK) as reported (3). 4-day cell cultures were used for attachment assays which were performed exactly as described (2, 3) using 96-well Linbro plates with flat bottoms (ICN Biomedicals Ltd, Bucks, UK). Attached cells were fixed with 1.25% glutaraldehyde, stained with 0.1% Toluidine blue and the absorbance at 492 nm was determined using an automatic microplate reader MRPA 4 (Eurogenetics, Belgium). For inhibition experiments, coated wells were incubated at room temperature for 30 min with 50 µl of appropriate dilutions of P3D4 or P1F11 mAbs prior to adding the cells.

ELISA assays. 96-well plates (Corning Glass Works, New York, USA) were coated with Fn (5 µg/well), 58 kDa (2 µg/well) or 38 kDa (1 µg/well) in PBS at 4° C overnight. Plates were rinsed with PBS, 0.05% Tween 20, and incubated with appropriate dilutions of mAbs. After 1 h at 37° C, the plates were rinsed with PBS/Tween and incubated for an additional hour with a 1:1000 dilution of peroxidase-conjugated goat IgG anti-mouse Igs (Dakopatts A/S, Glostrup, Denmark). The plates were washed and developed by addition of 1,2-Phenylenediamine dihydrochloride (OPD) in 0.1 M citric acid-phosphate buffer, pH 5.). After 30 min, the absorbance at 492 nm was determined using a microplate reader .

RESULTS

Biochemical characterization of mAbs P3D4 and P1F11 binding sites in fibronectin

To determine the specificity of the mAbs obtained using the immunization protocols described above, we performed ELISA assays with equal molar quantities of Fn, 80 kDa, 58 kDa, or 38 kDa fragments as substrata. Two mAbs termed P3D4 and P1F11 showed to be positive by this method. mAb P3D4 reacted in a dose dependent manner with fragments 38 and 58 kDa as well as with intact Fn (Fig. 1, left); P3D4 did not react with the 80 kDa fragment (not shown). mAb P1F11 was produced by immunizing with the synthetic peptide CS-1 and thus its reactivity with this peptide was not surprising (data not shown); however, it was important to determine whether this mAb recognized the CS-1 segment when present within Fn or Fn fragments. As shown in Fig. 1 (right), P1F11 recognized the 38 kDa fragment but not the 58 kDa fragment or intact Fn.

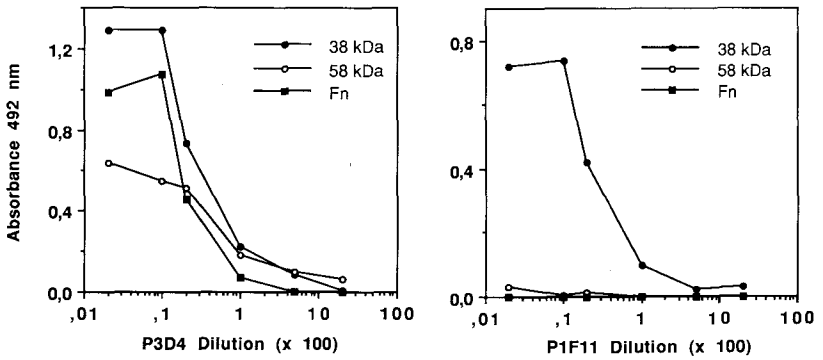

Figure 1. ELISA analysis of the reactivity of mAbs P3D4 and P1F11 with Fn and the 58 kDa and 38 kDa fragments. Wells were coated with Fn (5 µg), 58 kDa (2µg) or 38 kDa (1 µg) and incubated with the indicated dilutions of mAb P3D4 (left panel) or P1F11 (right panel). Quantitation of the reaction was done as described in Materials and Methods. Each determination was done in duplicate and values represent the average of two different experiments.

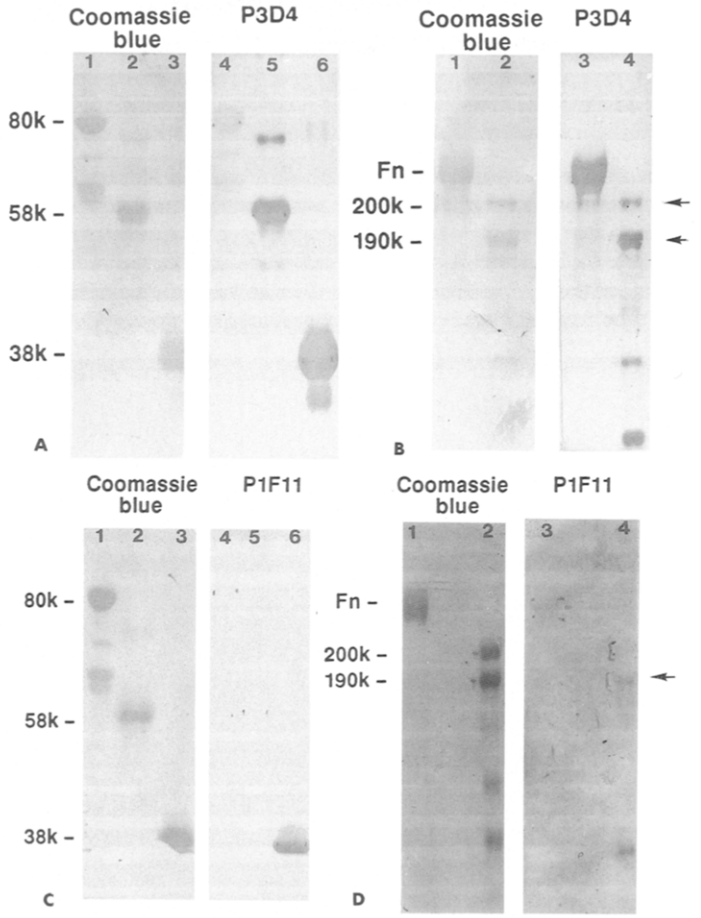

Figure 2. Western blot analysis of the reactivity of mAbs P3D4 and P1F11 with Fn and fragments. Panels A and C: nitrocellulose membrane transfers of a 10% acrylamide gel containing 80 kDa (lanes 1 and 4), 58 kDa (lanes 2 and 5), and 38 kDa (lanes 3 and 6) fragments. Membranes were stained with Coomassie blue (left panel) or incubated with either P3D4 (1:200 dilution, A) or P1F11 (1:500 dilution, C) (right panel); blots were developed with a peroxidase-labeled goat IgG anti-mouse Igs. Panels B and D: nitrocellulose membrane transfers of a 5% acrylamide gel containing Fn (lanes 1 and 3), and 200 kDa+190 kDa fragments (lanes 2 and 4). Membranes were stained with Coomassie blue (left) or incubated with mAbs (right) P3D4 (B) or P1F11 (D) and developed as above.

The results obtained by ELISA were confirmed by Western blots analyses of Fn and its fragments. As shown in Figure 2A, mAb P3D4 reacted with the 58 kDa (lane 5) and 38 kDa (lane 6) fragments, but not with the 80 kDa fragment (lane 4). P3D4 also reacted with intact Fn (Fig. 2B, lane 3) and with the two large tryptic fragments of 200 and 190 kDa (Fig. 2B, lane 4). These results indicate that mAb P3D4 recognizes an antigenic determinant common to both Hep II-containing fragments (58 and 38 kDa) and to both Fn chains (A and B). In contrast, mAb P1F11 (Fig. 2C) reacted with the 38 kDa fragment (lane 6), but failed to recognize

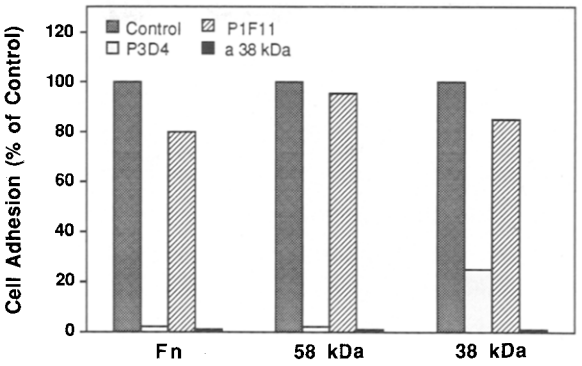

Figure 3. Effect of mAbs P3D4 and P1F11 on Ramos cell attachment to Fn or fragments. Wells were coated with 38 kDa (0.15 μg/cm^2), 58 kDa (5 μg/cm^2), or Fn (10 μg/cm^2) and incubated with P3D4 or P1F11 supernatants for 30 min, prior to adding the cells. Control wells (100% adhesion) are coated wells incubated with an irrelevant hybridoma supernatant. Attached cells were quantitated as previously described. Values are the average of three separate experiments.

the 58 kDa (lane 5) or 80 kDa (lane 4) fragments. These results clearly indicate that P1F11 recognizes a site present exclusively on the 38 kDa fragment. Based on molecular weights and specificity of trypsin cleavage, it was previously assumed that the 38 kDa and 58 kDa fragments are derived from the A and B chains of Fn respectively (1-3). If this is correct, the antigenic site recognized by P1F11 must be present in the A chain of Fn and absent from the B chain. To confirm this, we tested the reactivity of mAb P1F11 with the 200 and 190 kDa fragments. As shown in Figure 2D, P1F11 reacted with the190 kDa but not with the 200 kDa fragment (lane 4). mAb P1F11 gave a very faint reaction with intact Fn (lane 3), perhaps due to the relative molar quantity of the CS-1 segment in Fn as compared to the 38 kDa fragment. Another possible explanation is that CS-1 is not fully available when present in the intact molecule. Altogether these results clearly show that mAb P1F11 raised against the synthetic peptide CS-1, is able to recognize this site within the 38 kDa fragment and the A chain of Fn, and that the 58 kDa fragment lacks the CS-1 site and is derived from the B chain of Fn.

Functional properties of mAbs P3D4 and P1F11

We (1-3) and others (4, 7) have previously shown that the Hep II domain (contained in the 38 and 58 kDa fragments) and the CS-1 segment (contained in the 38 kDa fragment) of Fn support cell adhesion. We have also shown that the 38 kDa fragment is a more efficient substrate for lymphocyte attachment than the 58 kDa fragment (2, 3). The preceding results (Figs. 1-2) mapped the binding sites of mAbs P3D4 and P1F11 to the Hep II domain and the CS-1 site of Fn respectively. To further evaluate the contribution of both sites to lymphocyte attachment to Fn, we studied the adhesion of the B cell line Ramos to Fn and Fn fragments in the presence of mAbs P3D4 and P1F11. As shown in Figure 3, mAb

P3D4 efficiently inhibited (>95%) cell attachment to Fn or the 58 kDa fragment, and produced substantial inhibition (70-80%) of adhesion to the 38 kDa fragment. mAb P1F11 however produced only limited inhibition (10-20%) of adhesion to Fn or 38 kDa fragment (Fig. 3). mAb P1F11 completely inhibited cell attachment to the synthetic peptide CS-1 (not shown). Incubation with both mAbs simultaneously produced 95% inhibition of cell attachment to the 38 kDa fragment (not shown). For comparison, a polyclonal antibody anti-38 kDa fragment completely inhibited adhesion to Fn, 58 kDa and 38 kDa fragments (Fig. 3).

DISCUSSION

In this study we have obtained two new mAbs which recognize the Hep II and CS-1 sites in the carboxy-terminal region of Fn. We have used these mAbs to clarify the origen of Fn fragments derived from this region; since these two sites (Hep II and CS-1) are involved in cell attachment, we have also studied the effect of these mAbs on lymphocyte adhesion to Fn.

mAbs which specifically recognize the CS-1 site in Fn have not been previously reported. Two research groups have successfully obtained polyclonal antibodies to the IIICS region of Fn, using as immunogens a fusion protein containing a 95 amino acid residue stretch of IIICS (8), or a synthetic peptide comprising the last 31 residues of IIICS (9). Both of these segments (95 and 31 residues) are outside the CS-1 region studied here which comprises the first 25 amino acid residues of IIICS (3, 5). The results obtained with mAb P1F11 clearly show that the 58 kDa fragment is derived from the B chain of Fn. This was previously assumed (1-3, 7) based on the molecular weight of fragments derived from this region of Fn but can be definitely established now. Because the CS-1 segment is regulated by alternative splicing mechanisms, the availability of mAb P1F11 may be very useful to study the presence of the various Fn isoforms (with and without CS-1) in different tissues, particularly during development .

Although the precise site in Hep II recognized by mAb P3D4 was not mapped, the fact that this mAb efficiently inhibits cell adhesion to both 58 kDa and 38 kDa fragments and to Fn, suggests that the antigenic site recognized by P3D4 is directly involved (or in close proximity) in cell attachment. The finding that P3D4 is a good inhibitor of cell adhesion to the 38 kDa fragment was somewhat surprising, since this fragment contains two adhesion promoting sites (Hep II and CS-1) and CS-1 is apparently of higher affinity than Hep II (1-3). It is interesting

that mAb P1F11 (anti-CS-1) is a poor inhibitor of cell adhesion to the 38 kDa fragment or Fn. An explanation for this is that P1F11 does not bind native 38 kDa fragment (or Fn) with sufficient affinity to block cell attachment. Alternatively, P1F11 may bind to an epitope of CS-1 which is not directly involved in adhesion. In this regard, splitting CS-1 into the previously described A13 and B12 subpeptides (3) abolishes further binding to P1F11 (E. A. Wayner, unpublished), suggesting that the epitope recognized by this mAb is located in the middle of CS-1. It was recently shown (10, 11) that the active adhesive sequence within CS-1 is the tripeptide LDV located in the carboxy-terminal half of the peptide (within B12). A more likely explanation for the lack of inhibition of mAb P1F11 is that, contrary to the effect of P3D4, blocking CS-1 does not preclude cell binding to Hep II. The different functional properties of mAbs P3D4 and P1F11 suggest that the Hep II and CS-1 sites, may act in a cooperative manner to promote lymphocyte attachment to Fn. This suggestion is supported by the fact that both regions interact with the same cell receptor, the $\alpha 4\beta 1$ integrin (1, 3). It is therefore possible that interaction with Hep II is the first step in the process of cell adhesion to the carboxy-terminal region of Fn, and that this interaction regulates (and strengthen) binding to CS-1. While this mechanism awaits confirmation, the availability of the two novel mAbs described here would be very useful to fully understand the molecular bases and regulation of lymphocyte interaction with Fn.

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