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Mast cell adhesion to fibronectin

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SUMMARY

The MCP-5 murine mast cell line, as well as primary bone marrow-derived cultured mast cells (BMCMC), are demonstrated to bind to fibronectin, a ubiquitous adhesion protein of the extracellular matrix. BMCMC required activation by phorbol myristate acetate (PMA) to adhere to fibronectin, whereas MCP-5 displayed spontaneous adherence. The binding of both MCP-5 and BMCMC was dose dependent, with maximal adhesion at a fibronectin concentration of 20 μg/ml. The 120,000 molecular weight (MW) proteolytic fragment of fibronectin containing the RGDS cell attachment site was able to substitute for the native fibronectin molecule in promoting mast cell attachment. Mast cell adhesion to fibronectin, in addition, could be inhibited by the RGDS peptide alone. These data suggest that, in addition to the previously described mast cell–laminin interactions, mast cells also adhere to fibronectin, thus providing further insight into their tissue localization and possible roles in processes such as wound healing and fibrosis.

INTRODUCTION

The adhesion of cells to the extracellular matrix is important for many biological processes, such as embryogenesis,[1] tumour invasion and metastasis[2] and chemotaxis.[3,4] The discovery and characterization of the integrin family of cell-surface receptors has focused attention on the nature of these interactions between cells and the extracellular matrix. Integrins are heterodimers of two polypeptide chains (α and β) located within the cell membrane. Integrins recognize specific amino acid sequences within extracellular matrix proteins, as well as other cell-surface adhesion molecules, as reviewed elsewhere.[5] Specific recognition sites on proteins such as fibronectin, laminin, vitronectin or collagen allow the cell to attach to these macromolecules. This interaction between the cell and its surrounding matrix is capable of inducing a number of biological responses. Perhaps the most visible example of the cellular response to this receptor–ligand interaction is the re-organization of the cytoskeleton, change of cell shape, and spreading which occurs when a variety of cell types are exposed to surfaces coated with extracellular matrix proteins.[6-8] In addition, the interaction of integrin receptors with their ligands can generate signals which result in the induction of new genes[9] and which affect the process of cell differentiation.[10]

The mast cell is a haematopoietically derived cell best known for its effector role in atopic diseases such as asthma and anaphylaxis. Although a clear role for the mast cell in homeostasis has yet to be defined, activated mast cells are capable of releasing or generating a number of biologically active mediators. Despite their haematopoietic origin, mature mast cells are not found in the blood. Rather, mature mast cells are found almost exclusively in tissues; however, to date the only documented interaction between connective tissue components and mast cells has involved laminin. Mast cells have recently been shown to attach to and spread on laminin following activation with PMA or following crosslinking of the high-affinity IgE receptor.[11,12] Because of the probability that specific cellular responses are generated *in vivo* as cells adhere to multiple matrix components either simultaneously or in sequence,[13] we chose to examine whether mast cells might not also adhere to fibronectin. In this report, we demonstrate the specific adherence of mast cells to fibronectin and that adherence is increased following mast cell activation. Further, we show that mast cell binding to fibronectin appears to involve the RGD sequence located within the cell-attachment domain of the fibronectin molecule.

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MATERIALS AND METHODS

Materials

Bovine fibronectin, rabbit anti-bovine fibronectin antiserum, and rabbit anti-bovine serum albumin antiserum (Chemicon International Inc., Temecula, CA); human fibronectin (Boehringer-Mannheim, Indianapolis, IN); chymotryptic fragments of human fibronectin (a 40,000 MW fragment containing heparin binding site, a 45,000 MW fragment containing gelatin binding site, and the 120,000 MW fragment containing a cell-attachment site; Calbiochem, San Diego, CA); WEHI-3-conditioned medium (Collaborative Research Inc., Bedford, MA);

and Immulon 96-well plates (Dynatech Laboratories Inc., Chantilly, VA) were purchased from the manufacturers. Purity of the fibronectins was verified by SDS-PAGE electrophoresis in reducing conditions.[14]

Mast cell cultures

The mast cell line MCP-5[15] was maintained in RPMI-1640 media supplemented with 25 mM HEPES, 4 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 0·1 mM non-essential amino acids and 1 mM sodium pyruvate (complete RPMI) with 10% heat-inactivated foetal calf serum (FCS) and 10% WEHI-3-conditioned media as a source of interleukin-3 (IL-3). Cells were harvested weekly, with remaining cells resuspended in fresh media at 5×10^5 cells per ml. Primary cultures of mast cells (BMCMC) were obtained from 5-week cultures of mouse bone marrow (taken from the femurs of 6-week-old BALB/c female mice). Cells were maintained in complete RPMI-1640 media supplemented with 10% FCS and 10% WEHI-3-conditioned media at a concentration of 2×10^5 cells per ml. Such 5-week-old bone marrow cultures consisted of greater than 95% mast cells as determined by light microscopic examination of haematoxylin and eosin-stained cytospin preparations.

Cell adhesion assay

Adherence was determined using radiolabelled cells.[16,17] Mast cells were centrifuged at 800 ***g*** for 10 min at room temperature and resuspended in complete RPMI with 10% FCS and 10% WEHI-3-conditioned media at a final concentration of 1×10^6 cells per ml. [^{3}H] thymidine was added to a final concentration of 5 μCi/ml for MCP-5 mast cells or 10 μCi/ml for BMCMC and the cells incubated for 16 hr in a CO_2 incubator. Cells were then centrifuged at 800 ***g*** for 10 min at room temperature, resuspended in complete RPMI, centrifuged, and resuspended in complete RPMI (without FCS or WEHI-3-conditioned medium) at 4×10^5 cells per ml prior to use in adhesion experiments. Phosphate-buffered saline (PBS) (0·1ml) containing bovine or human fibronectin or chymotryptic fragments of human fibronectin at concentrations indicated for specific experiments was added to each assay well and incubated overnight at room temperature. Control wells were incubated with PBS alone. Four per cent bovine serum albumin (BSA) in PBS (0·1 ml) was next added to each well and the plates incubated for 2 hr at 37° to inhibit non-specific binding to polystyrene.[18] The wells were then gently rinsed twice with 0·1 ml complete RPMI, and 4×10^4 mast cells in 0·1 ml complete RPMI were added to each well. To determine the effect of phorbol myristate acetate (PMA), PMA dissolved in complete RPMI was added to selected wells at a final concentration of 50 ng/ml. This concentration of PMA did not release histamine from MCP-5 or BMCMC. The multi-well plates were then placed in a CO_2 incubator for 90 min, unless stated otherwise. After incubation, the liquid cell suspension was removed from each well separately using a multi-channel pipette and transferred to a new 96-well plate. Each well of the original plate (containing the adherent cells) was washed twice with 0·2 ml complete RPMI, and these wash fluids added to the cell suspension that had been transferred to the new plates. Radioactivity in the non-adherent cell population was determined by harvesting the initial cell-containing supernatant and the two corresponding wash fluids through the same harvesting filter (PHD cell harvester, Cambridge Technology, Inc., Cambridge, MA). Radioactivity remaining with adherent cells was determined by washing the wells through 15 cycles using water as the rinsing fluid on a new filter membrane. The filter-bound radioactivity was measured in a liquid scintillation counter (Tri-Carb 2200 CA; Packard Instrument Company, Downers Grove, IL) using Hydrofluor (DuPont, Wilmington, DE) as the scintillation fluid. The radioactivity in the adherent and non-adherent cells was considered an index of cell number and the percentage of cell adhesion calculated by the following formula:

$$\% \text{ cell adhesion} = \frac{\text{radioactivity in adherent cells}}{\text{radioactivity in adherent cells} + \text{radioactivity in non-adherent cells}} \times 100.$$

It was found in preliminary experiments that radioactivity incorporated per adherent cell was equal to the radioactivity incorporated per non-adherent cell (data not shown).

Inhibition of cell adhesion

To determine the effect of anti-bovine fibronectin antiserum, wells coated with 20 μg/ml bovine fibronectin were incubated for 1 hr at room temperature with 0·1 ml antiserum diluted from 1:10 to 1:100 in PBS. Wells were then rinsed twice with 0·1 ml complete RPMI and used in cell adhesion assays. As a control, the fibronectin-coated albumin-blocked wells were incubated with either media alone or with an anti-bovine albumin antiserum diluted 1:10. In a separate experiment, 10 μl RGDS peptide diluted in PBS at concentrations ranging from 0 to 2000 μg/ml were added just after the addition of radioactively labelled MCP-5 or BMCMC cells to wells coated with 20 μg/ml human fibronectin. Cells were then incubated for 90 min and percentage adherence determined as described above.

RESULTS

A number of haematopoietic cells, including neutrophils and macrophages, can be shown to adhere to fibronectin under appropriate conditions.[13,19,20] To determine if mast cells could adhere to fibronectin, we first examined cultures of mast cells placed on fibronectin-coated surfaces by light microscopy. As shown in Fig. 1a, MCP-5 cells did not adhere to plastic surfaces coated with bovine serum albumin (BSA) alone. In contrast, MCP-5 cells were found to adhere to fibronectin-coated surfaces both in the absence (Fig. 1b) and presence (Fig. 1c) of PMA. BMCMC also did not adhere to surfaces coated with BSA alone but, unlike the MCP-5 cell line, they did not display any appreciable spontaneous adherence to fibronectin; however, adherence to fibronectin could be induced by activation of the cells by PMA (Fig. 1d).

To further characterize mast cell–fibronectin interactions, we employed an assay using mast cells radiolabelled with [^{3}H] thymidine.[16,17] As seen in Fig. 2, MCP-5 mast cells spontaneously attached to both bovine (a) and human (b) fibronectin, with maximal attachment to bovine fibronectin at a concentration of 20 μg/ml and to human fibronectin at a concentration of 10 μg/ml. PMA activation led to a 111% increase in mast cell attachment at 20 μg/ml bovine fibronectin and an 83% increase at 20 μg/ml human fibronectin. Initial observations (Fig. 1d) demonstrated that primary BMCMC would also adhere to fibronectin in the presence of PMA. As shown in Fig. 3, primary BMCMC cultures differed from MCP-5 mast cells in that

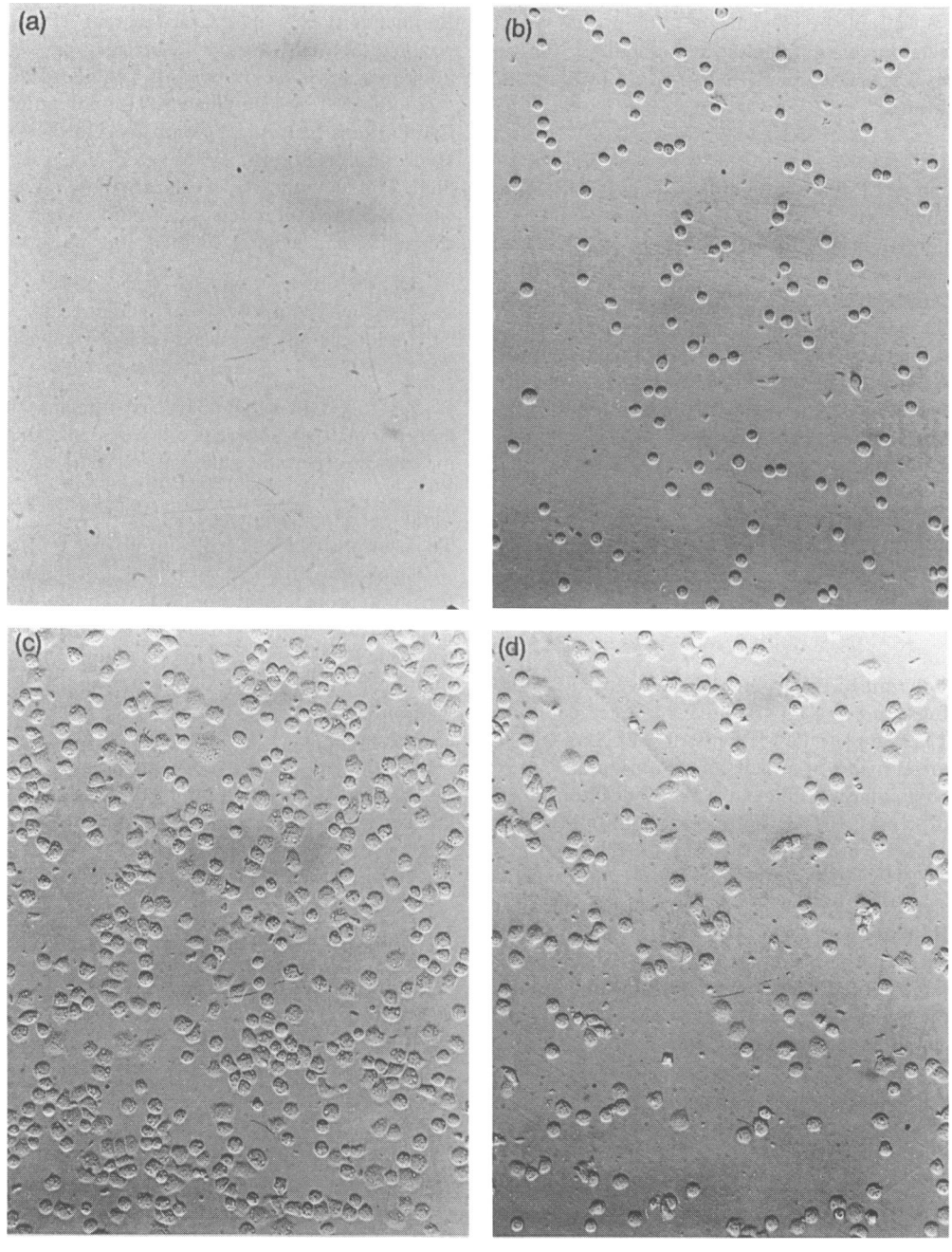

Figure 1. Mast cell adhesion on a fibronectin substratum. Adhesion of MCP-5 mast cells in plastic wells in the absence of fibronectin (a), or to wells coated with 20 μg/ml fibronectin in the absence (b) or presence (c) of 50 ng/ml PMA; (d) shows BMCMC adhering to plastic wells coated with 20 μg/ml fibronectin in the presence of 50 ng/ml PMA. Observations were made 60 min after the addition of mast cells. For details, see the Materials and Methods. Wells were photographed at 200 × using an inverted microscope (Zeiss Axiovert 405 M) with Hoffman optics.

virtually no attachment to fibronectin was observed in the absence of PMA.

We next examined the time-course of MCP-5 adherence to fibronectin-coated surfaces (Fig. 4). PMA-activated MCP-5 cells rapidly attached to fibronectin-coated surfaces, with approximately 50% of maximal attachment achieved within the first 10 min. Maximal attachment was observed at approximately 90 min after addition of cells to the fibronectin-coated wells. In the absence of PMA, although 40% of the cells were adherent at 30 min, the number of adherent cells decreased to approximately 20% by 60 min after exposure to the fibronectin-coated surface.

To verify that the observed adherence was specific for fibronectin, anti-fibronectin antibody was added to the fibronectin-coated wells prior to the addition of cells and the percentage cell adherence determined. As can be seen in Fig. 5, addition of anti-fibronectin antibody significantly decreased both spontaneous cell adhesion to fibronectin as well as that observed after activation with PMA. For example, a 1:20 dilution of anti-fibronectin antibody decreased adhesion com-

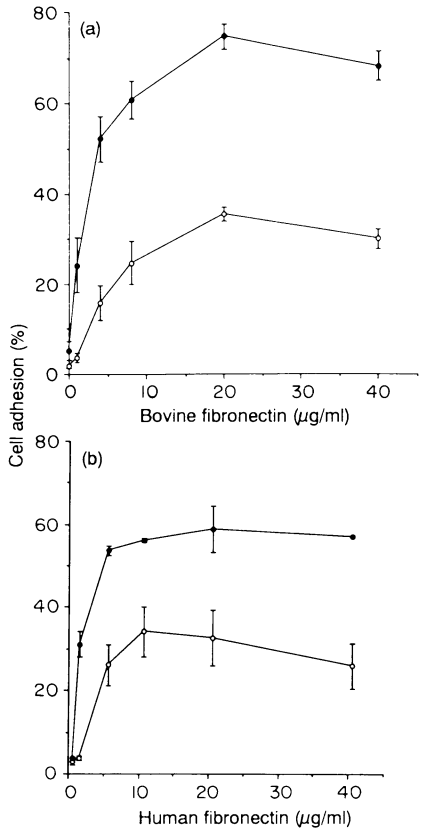

Figure 2. Adhesion of MCP-5 mast cells to fibronectin-coated plastic. Mast cells were added to wells coated with increasing amounts of (a) bovine fibronectin or (b) human fibronectin in the presence (solid circles) or absence (open circles) of 50 ng ml PMA. Values represent mean ± SEM for five independent experiments, each of which was performed in duplicate.

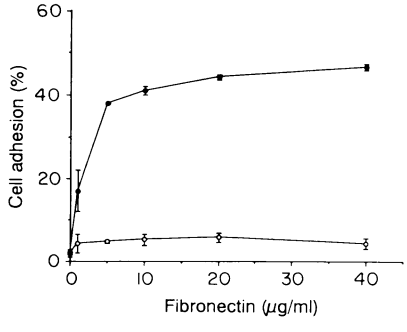

Figure 3. Adhesion of BMCMC to fibronectin-coated plastic. Dose-dependent adhesion to human fibronectin in the presence (solid circles) or absence (open circles) of 50 ng ml PMA. Values represent mean ± SEM for four independent experiments, each of which was performed in duplicate.

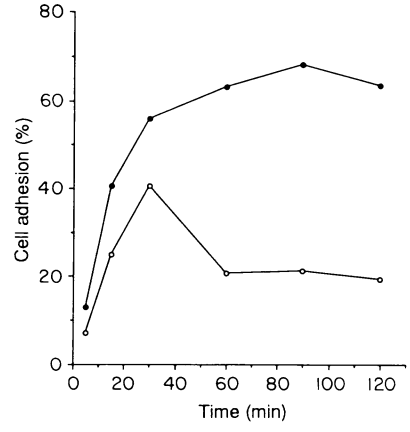

Figure 4. Kinetics of MCP-5 mast cell adhesion to fibronectin. Mast cells were incubated for the time periods indicated on the graph in wells coated with human fibronectin (20 μg/ml) in the presence (solid circles) or absence (open circles) of PMA (50 ng ml). The adhesion of BSA-coated control wells was determined for every time-point and subtracted. Adhesion to control wells in every instance was less than 5%. Values represent the mean for two separate experiments, each of which was performed in duplicate.

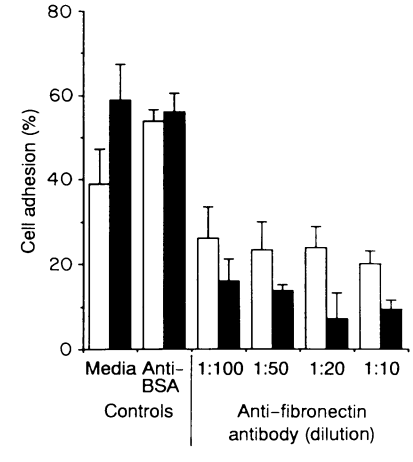

Figure 5. Inhibition of MCP-5 mast cell adhesion by anti-fibronectin antiserum. Wells coated with bovine fibronectin were incubated with anti-bovine fibronectin antiserum (see the Materials and Methods) followed by the addition of cells in presence (solid bars) or absence (open bars) of PMA (50 ng ml). Controls consisted of fibronectin-coated wells incubated with media alone (Media) or fibronectin-coated wells incubated with anti-bovine albumin antiserum diluted 1:10 (Anti-BSA). Values represent mean ± SEM for three independent experiments, each of which was performed in duplicate.

pared to the media control by 86% in PMA-treated cells and by 39% in cells not exposed to PMA.

We next sought to determine which portion of the fibronectin molecule was responsible for binding to mast cells. The plastic surface was first coated with equimolar concentrations of either native fibronectin or one of three fibronectin chymotryptic fragments. These included a 40,000 MW fragment containing the heparin binding site, a 45,000 MW fragment

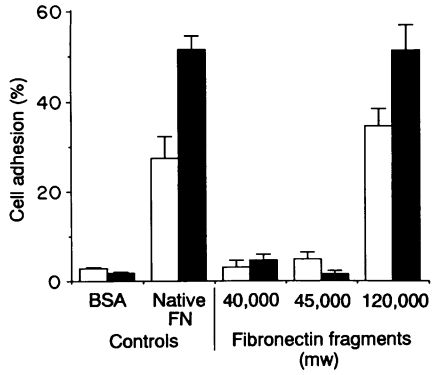

Figure 6. Adhesion of MCP-5 mast cells to chymotryptic fragments of human fibronectin. Plastic wells were coated with equimolar concentrations of 40,000, 45,000 or 120,000 MW fibronectin fragments. Control wells were coated with native fibronectin (Native Fn) or BSA only. Experiments were performed in the presence (solid bars) or absence (open bars) of 50 ng/ml PMA. Values represent mean ± SEM for three independent experiments, each of which was performed in duplicate.

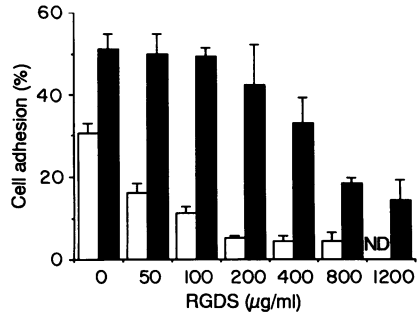

Figure 7. Inhibition of MCP-5 mast cell adhesion by RGDS peptide. The graph shows adhesion of mast cells to human fibronectin (20 μg/ml) in the presence of RGDS in concentrations indicated on the graph in the presence (solid bars) or absence (open bars) of 50 ng/ml PMA. Values represent mean ± SEM for three independent experiments, each of which was performed in duplicate. ND, not determined.

containing a gelatin binding site, or a 120,000 MW fragment containing the known RGD tripeptide cell attachment site. As is shown in Fig. 6, both unstimulated MCP-5 cells and those activated by PMA were found to attach to the 120,000 MW fragment of fibronectin, but not to 35,000 or 40,000 MW fragments. In both cases, the percentage of cells adhering to the plastic coated with the 120,000 MW proteolytic fragment was equal to that observed with the native fibronectin molecule, suggesting that this region of the molecule was responsible for the entirety of the observed adhesion.

The RGDS peptide has been shown previously to inhibit integrin-mediated cell adhesion to fibronectin. To determine if this peptide could similarly inhibit mast cell attachment to fibronectin, increasing amounts of the RGDS peptide were added to the fibronectin-coated wells simultaneously with the addition of cells. As can be seen in Fig. 7, cell adhesion to fibronectin in the absence of PMA was inhibited by 80% at 200 μg/ml of RGDS peptide. In contrast, adherence of PMA-stimulated mast cells could also be inhibited by the RGDS

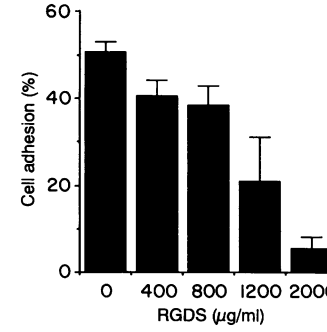

Figure 8. Inhibition of BMCMC adhesion by RGDS peptide. Adhesion of PMA-activated (50 ng/ml) BMCMC to human fibronectin (20 μg/ml) in the presence of RGDS in concentrations indicated on the graph. Values represent mean ± SEM for three independent experiments, each of which was performed in duplicate. BMCMC did not attach to fibronectin in the absence of PMA (see Fig. 3).

tetrapeptide, although much higher concentrations were required, with 1200 μg/ml RGDS peptide needed to inhibit mast cell adhesion by 72%. For BMCMC, maximal attachment occurred at a concentration of fibronectin (20 μg/ml) similar to that which gave maximal attachment for MCP-5. The attachment of BMCMC to fibronectin was also inhibited by RGDS peptide (Fig. 8).

DISCUSSION

A number of diverse cell types, including fibroblasts, macrophages, osteosarcoma cells and neutrophils, have been reported to adhere to fibronectin.[13,19,20] The data presented in this paper clearly demonstrate that murine mast cells also adhere to fibronectin. Thus, under proper conditions, both the MCP-5 mast cell line and primary cultures of murine mast cells adhere to fibronectin (Figs 2 and 3); however, primary BMCMC adhere to fibronectin only after activation with PMA, which enhances, but is not required for, the adherence of MCP-5 mast cells to fibronectin. Cloned cell lines frequently display an activated state due to maintenance in culture,[21] which may explain the spontaneous adhesion of the MCP-5 cell line to fibronectin, whereas BMCMC represent a resting cell population. In all likelihood, cross-linking of high-affinity IgE receptors on BMCMC would also be capable of inducing activation of cell adherence to fibronectin, as has been observed with laminin.[11] A similar enhancement of cell attachment is also seen when fibroblasts are exposed to PMA.[22] Also as with fibroblasts, adherence of mast cells to fibronectin is accompanied by spreading of the cells over the fibronectin-coated surface (Fig. 1).

It is known that human fibroblasts adhere to fibronectin through the classical fibronectin integrin receptor VLA-5 composed of the $\alpha_5\beta_1$ subunits.[13] This adhesion to fibronectin involves the cell attachment site on the fibronectin molecule and can be blocked by RGDS peptide.[23,24] In addition, the α_v subunit of the vitronectin receptor ($\alpha_v\beta_3$) has been shown to associate with two other β subunits, resulting in the formation of receptors with fibronectin specificities.[25,26] The alternative β subunits include β_1 (producing a $\alpha_v\beta_1$ receptor that binds fibronectin but not vitronectin) present on fibroblast and

neuroblastoma cell lines[26] and a β_5 subunit producing $\alpha_v\beta_5$ on epithelial cells and some carcinomas, which continues to bind vitronectin, now binds fibronectin, but loses specificity for fibrinogen.[25] These latter two receptors also demonstrate RGDS peptide sensitivity; however, not all integrins bind to fibronectin via RGD-containing domains. For example, the $\alpha_4\beta_1$ receptor binds to the variable region (CS-1) of fibronectin which does not contain an RGD sequence.[27] The attachment of murine mast cells to fibronectin likewise involves the 120,000 MW polypeptide containing the cell attachment site (Fig. 6). Additionally, both the attachment of MCP-5 mast cells and BMCMC to fibronectin are inhibited by RGDS peptide (Figs 7 and 8). These data suggest that mast cell adherence to fibronectin is through an integrin receptor. Unfortunately, there is little information on the integrin receptor on murine cells. A 135,000 MW membrane glycoprotein on mouse fibroblasts has been postulated to be a possible fibronectin receptor.[28] Recently, the fibronectin receptor on mouse macrophages was cloned and appeared to be the murine equivalent of human VLA-5.[29] Studies are under way in our laboratory to determine if mast cell adhesion to fibronectin is, indeed, mediated by a receptor from the integrin family.

To date, laminin is the only substance for which mast cell adherence has been demonstrated. Similar to the data presented here for fibronectin, mast cell attachment to laminin is also increased following activation with PMA. Interestingly, the initial paper describing attachment to laminin did not observe mast cell attachment to fibronectin.[11] This failure to detect mast cell adhesion to fibronectin in the earlier study may have been a result of differences in the cell adhesion assay employed. These include: type of plastic, conditions during the coating procedure, and absence of FCS in the system; however, using a cell adhesion assay successful in other experimental systems,[16,17] we found substantial adhesion of mast cells to fibronectin. It is not only possible but probable that additional substrates for mast cell adhesion will be identified based on the experience in other inflammatory cell types where a number of specific adhesion molecules seem to be involved in a variety of cellular functions.

The ability of mast cells to adhere to fibronectin may play a role in the migration of mast cells in tissue. This may be particularly important in areas of fibronectin deposition, including those which occur at wound sites where mast cells are known to increase in number. As has been described, the mast cell–laminin interaction may also alter gene expression and thus modify the biological responses of mast cells.[30] Studies are under way to both delineate the mast cell fibronectin receptor and to determine if adherence of mast cells to fibronectin alters mast cell biology.

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