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Role of β1 Integrins in Adhesion of Canine Mastocytoma Cells to Extracellular Matrix Proteins

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ABSTRACT. The interactions of tumor cells with the extracellular matrix (ECM) are a crucial step in invasion and metastasis. Integrins are adhesive molecules forming heterodimers with α and β subunits that play a definitive role in these interactions. In this study, mastocytoma (mast cell tumor: MCT) cell-ECM interaction was investigated using 3 canine MCT cell lines: CM-MC (originating from cutaneous MCT), VI-MC (originating from intestinal MCT), and CoMS (originating from oral MCT). Flow cytometric analysis showed that all cells highly expressed the integrin β1 and α1 through α5 subunits and that they moderately expressed the α6 subunit. In adhesion studies, CoMS weakly but spontaneously adhered to fibronectin (FN), which was enhanced by phorbol ester (TPA), while CM-MC and VI-MC required cell activation by TPA to adhere to FN. Anti-β1 and α5 integrin antibodies strongly inhibited cell adhesion to FN in CM-MC and CoMS and moderately inhibited cell adhesion in VI-MC. Only VI-MC adhered to laminin (LN) under activation by TPA. Anti-β1 integrin antibodies strongly inhibited cell adhesion to LN, but all anti-α integrin antibodies failed to inhibit cell adhesion to LN. No cells adhered to collagen types I and IV. Canine MCT cells from different origins expressed similar integrin patterns; however, there were some differences in adhesive behavior in response to various ECM proteins and activating stimuli.

KEY WORDS: canine, extracellular matrix, integrin β1 subfamily, mastocytoma cell.

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Canine mast cell tumors (MCTs) account for between 16 and 21% of all cutaneous tumors and are highly invasive and metastatic depending on their histological grade. Canine MCTs infrequently develop in other sites, including the oral cavity and intestines, which are associated with poorer prognoses [18, 20].

Tumor invasion and metastasis are multistep biological processes in which the interactions of tumor cells with extracellular matrix (ECM) components are believed to play an important role. These interactions are partly mediated via a family of cell-surface receptors called integrins that specifically recognize a number of ECM proteins [1, 7]. The integrin receptor family comprises over 20 distinct αβ heterodimeric transmembrane glycoproteins, in which the association between the α and β subunits determines the ligand-binding specificity of the receptor molecule. There is massive variability in their functions among different cell types, and it has been reported that conversion to a migratory and invasive phenotype is related to changes in integrin expression, intracellular control of integrin functions, and signals perceived from integrin ligand binding [13].

Although canine mastocytoma (MCT) cells have been reported to weakly adhere to fibronectin (FN) and collagen (Coll), the role of integrin in adhesion has not been clearly elucidated [22]. In the present study, we evaluated whether canine MCT cell lines expressed the integrin β1 subfamily, which is potentially involved in adhesion to ECM proteins, and studied the properties of MCT cell adhesion to the ECM.

MATERIALS AND METHODS

Cell lines: Three canine MCT cell lines from different origins, CM-MC (originating from cutaneous MCT), VI-MC (originating from intestinal MCT) and CoMS (originating from oral MCT), were used in this study. They were maintained in RPMI 1640 culture medium with 10% fetal bovine serum (FBS) as described previously [8, 19].

Extracellular matrix components: The ECMs used were FN (bovine FN, Yagai, Yamagata, Japan), laminin (LN; mouse LN, BD Biosciences, San Jose, CA, U.S.A.), Coll type I (Coll I; derived from swine skin, Wako Pure Chemical Industries, Osaka, Japan, and from bovine skin, Sigma Chemical Co., St. Louis, MO, U.S.A.), and Coll type IV (Coll IV; derived from bovine lens, Wako, and from human placenta, Sigma).

Antibodies: The following antibodies (Abs) were used to analyze the expression and function of integrins. Mouse monoclonal Ab (mAb) to human CD29 (integrin β1, clone 4B4, mouse IgG1) was purchased from Beckman Coulter, Fullerton, CA, U.S.A. Mouse mAbs to human CD 49a (integrin α1, clone HP2B6, mouse IgG1), human CD 49b (integrin α2, clone Gi9, mouse IgG1), human CD 49d (integrin α4, clone HP2/1, mouse IgG1), human CD 49e (integrin α5, clone SAM1, mouse IgG2b), and rat mAb to mouse CD 49f (integrin α6, clone GoH3, rat IgG2a) were purchased from BD Biosciences. Mouse mAb to human CD 49c (integrin α3, clone P1B5, mouse IgG1) was purchased

from Immunotech, Marseille, France. The mAbs 4B4, HP2/1, SAM1 [16], HP2B6, Gi9 [9], P1B5 [24], and GoH3 [17] have previously been shown to possess inhibitory activity against each ligand in cell adhesion assays. FITC-conjugated goat antisera to mouse IgG and FITC-conjugated goat Ab to rat IgG $F(ab')_2$ were purchased from MP Biomedicals, Irvine, CA, U.S.A. Purified mouse and rat IgG Abs (Chemicon International, Temecula, CA, U.S.A.) were used as isotype-matched control Abs.

Flow cytometric analysis: Expression of the integrin receptors of the cell surface was analyzed by flow cytometry as follows. Briefly, 5×10^5 cells were fixed with 5% paraformaldehyde for 10 min at 4°C and preincubated for 30 min at 4°C with 10% normal goat serum in order to block nonspecific binding sites. The cells were incubated with saturated concentrations of primary Abs overnight at 4°C, incubated with the second Abs for 1 hr at 4°C, and analyzed using a FACScan flow cytometer (Nippon Becton Dickinson Company, Tokyo, Japan). Cells that were not incubated with primary Abs were considered to be the negative control.

Adhesion assay: For adhesion assay, flat-bottomed wells in microtiter plates (Multi Well Plates, Sumitomo Bakelite, Tokyo, Japan) were coated overnight at 4°C with 100 μl of each ECM diluted to 100 μg/m*l* with serum-free RPMI 1640 medium. The plates were rinsed with phosphate buffered saline (PBS), and nonspecific binding sites were blocked by incubation with 100 μl of RPMI 1640 with 1% bovine serum albumin (BSA) for 1 hr at 37°C. The plates were rinsed again, and a total of 1×10^5 cells in 100 μl of RPMI 1640 medium with 0.1% BSA were plated in triplicate on each coated well and incubated for 1 hr at 37°C. In order to determine nonspecific binding, cells were also added to wells that were coated with 3% BSA (control). Unbound cells were removed by gentle washes with RPMI 1640 medium without FBS. The percentage of adherent cells in control wells was always less than 5%. The number of adherent cells was counted, and the percentage of adherent cells was calculated as follows:

% adhesion = (number of ECM-adherent cells – number of BSA-adherent cells) / (total number of cells seeded – number of BSA-adherent cells) × 100. To assess the effect of protein kinase C (PKC) activation on cell adhesion, cells were preincubated with phorbol ester (TPA; 12-*O*-tetradecanoylphorbol-13-acetate, Sigma, final concentration of 10 *n*g/m*l*) for 30 min at 37°C before being plated onto the ECM-coated plates in some experiments.

Inhibition assay: To determine which integrin chain on the cells was involved in binding to each ECM protein, the adhesion assay described above was performed using blocking Abs specific for each integrin chain. Cells were preincubated with mAbs (1 μg/m*l*) or isotype-matched control Abs (1 μg/m*l*) for 30 min at 37°C, and the adhesion assay was performed under the presence of each Ab. To determine the effect of peptides, including tripeptide arginine-glycine-aspartic acid (RGD; the RGD consensus sequence within FN binds to a subset of integrins), on cell adhesion, cells were preincubated with a synthetic peptide (arginine-glycine-aspartic acid-serine; RGDS peptide, Sigma) at a final concentration of 250 μg/m*l* for 30 min at 37°C before the adhesion assay was conducted. Arginine-glycine-glutamine-serine (RGES peptide, Sigma) was used as an inactive control peptide. The number of ECM-adherent cells without Abs or peptide was defined as representing 100% adhesion (control).

Statistical analysis: In flow cytometric analysis, all measurements were repeated at least 3 times. Results were expressed as the mean value of the percentage of positively stained cells. The adhesion and inhibition assays were performed in triplicate and were repeated at least 3 times. The results were expressed as means ± SD. One-factor ANOVA followed by Scheffé's multiple comparison test was used for comparison of differences between measured values and control values. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Expression of β1 integrins: Flow cytometry showed a similar expression pattern for all integrin subunits belonging to the integrin β1 subfamily for each cell line (Table 1). All cell lines showed high expression of the integrin β1 and α1 through α5 subunits (nearly 100%) and moderate expression of the integrin α6 subunit. There was little difference in the integrin expression levels of the cell lines.

Adhesion to ECMs: Although 3 cell lines showed similar expression patterns for the integrin subunits, which are putative receptors for FN, LN, and Coll, their patterns of adhesion were different. In the adhesion assay, the CM-MC and VI-MC cell lines adhered significantly to FN only after activation with TPA (19.3 ± 2.8% and 45.0 ± 6.5%, respectively; Fig. 1). On the other hand, CoMS showed spontaneous adhesion to FN (14.8 ± 6.0%), and the degree of adhesion was enhanced by TPA activation (51.3 ±4.5%). None of the cell lines spontaneously attached to LN; however, VI-MC attached to LN under TPA activation (36.1 ± 4.6%). There was no significant cell adhesion to Coll I and IV in any cell lines regardless of TPA activation. Cell lines

Table 1. Expression of cell surface molecules on mastocytoma cells, as determined by flow cytometry

Integrin subunit	ECM ligand[a)]	% expression[b)] CM-MC	VI-MC	CoMS
β1		98.6	99.0	98.4
α1	LN, Coll	99.7	99.4	98.4
α2	LN, Coll	99.6	99.9	99.9
α3	FN, LN, Coll	99.9	99.1	99.8
α4	FN	99.9	99.5	99.9
α5	FN	99.7	100.0	100.0
α6	LN	56.9	55.4	70.0

a) The major extracellular matrix (ECM) ligands of these adhesion receptors are as follows: LN, laminin; Coll, collagen; and FN, fibronectin.

b) Values are given as means (n≥3) of the percentages of positively stained cells

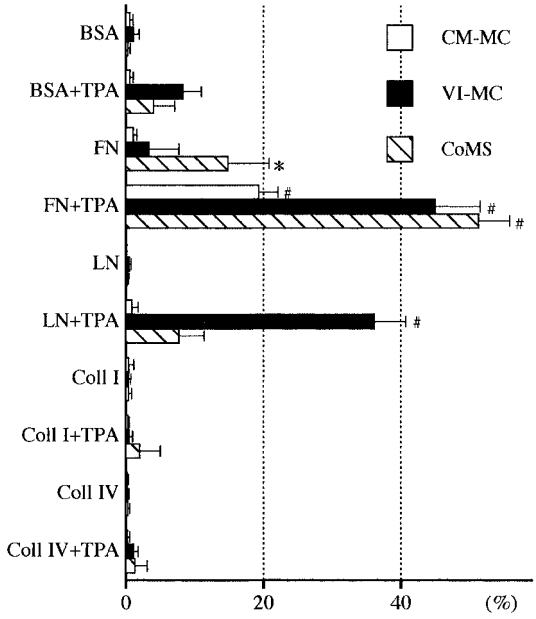

Fig. 1. Adhesion rates of canine mastocytoma cells to extracellular matrix (ECM) proteins. Cells were incubated for 1 hr in wells coated with different ECM proteins in the presence or absence of phorbol ester (TPA). Each value represents the mean ± SD of 3 experiments. * $P<0.05$ indicates a significant difference compared with the controls (3% bovine serum albumin [BSA]). # $P<0.05$ indicates a significant difference compared with the controls (3% BSA) + TPA. FN, fibronectin; LN, laminin; Coll, collagen.

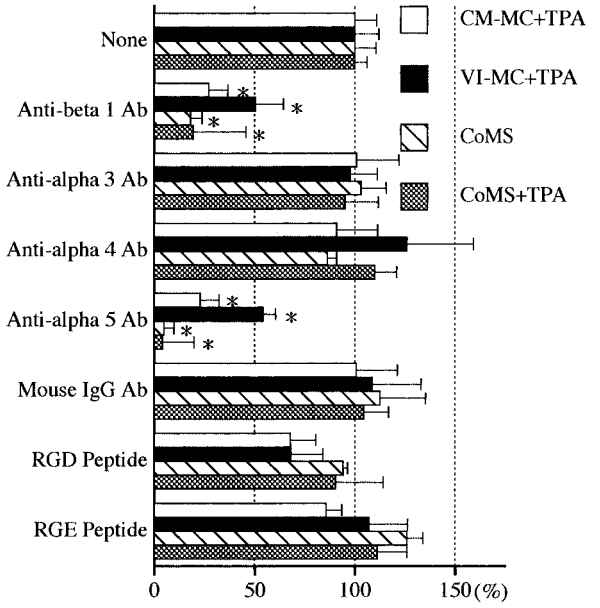

Fig. 2. Effect of anti-integrin antibodies (Abs) and synthetic peptide on the adhesion of canine mastocytoma cells to fibronectin (FN). Cells were incubated with specific Abs or control Ab (mouse IgG Ab) and a synthetic peptide (RGD) or control peptide (RGE) in the presence or absence of phorbol ester (TPA). Results represent means ± SD of 3 experiments. * $P<0.05$ indicates a significant difference compared with the controls (None, without any Ab). The number of FN-adherent cells without Ab or peptide with or without TPA is defined as representing 100% adhesion.

also did not attach to any Coll products from human, swine, and bovine origins at any concentrations (data not shown).

Integrin subunits specific to ECM binding: Based on the results of the adhesion assay, inhibition assay was performed when cells significantly adhered to ECM. Since adhesion to FN is mediated via integrins $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$, we conducted the inhibition assay using neutralizing mAbs against these integrin subunits (Fig. 2). Among the Abs for each integrin subunit, the Abs against the $\beta1$ and $\alpha5$ subunits inhibited adhesion. When the Ab against $\beta1$ subunit was used, the adhesion rate to FN significantly decreased to 27.1 ± 9.5% in CM-MC with TPA, 50.3 ±14.0% in VI-MC with TPA, 18.0 ± 5.7% in CoMS, and 19.2 ± 26.4% in CoMS with TPA, respectively, compared with the controls. When the Ab against $\alpha5$ subunit was used, the adhesion rate to FN significantly decreased to 22.9 ± 9.4% in CM-MC with TPA, 54.0 ± 6.4% in VI-MC with TPA, 4.8 ± 4.9% in CoMS, and 4.0 ± 15.7% in CoMS with TPA, respectively, compared with the controls. The Abs against the $\alpha3$ and $\alpha4$ subunits did not significantly suppress adhesion to FN. When a synthetic peptide that competed with the RGD sequence of FN was used, adhesion to FN was not significantly inhibited in any of the MCT cells.

Since adhesion to LN is mediated via integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$, an inhibition assay was performed using neutralizing mAbs against these integrin subunits (Fig. 3). VI-MC with TPA activation was used since it was the only cell line to show significant adhesion to LN. Neutralizing the $\beta1$ subunit significantly inhibited adhesion to LN; the adhesion rate fell to 21.6 ± 9.0% compared with the controls. None of the Abs against the α subunits suppressed adhesion to LN.

DISCUSSION

Cell adhesion to ECM proteins is predominantly mediated by integrin, which may play key roles in the regulation of tumor invasion and metastasis [1, 7, 13]. The present study showed that canine MCT cells strongly express the integrin $\beta1$ subfamily and that they are capable of binding to ECM proteins. Moreover, our inhibition studies showed that mast cell adhesion to FN and LN is mediated, at least in part, by the integrin $\beta1$ subfamily.

In humans, it has been reported that the expression pattern of the integrin $\beta1$ subfamily and the degree of expression varies depending on the origin of the mast cells. For example, uterine mast cells express integrins $\alpha4\beta1$ and $\alpha5\beta1$, skin mast cells express integrins $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$, and intestinal mast cells express integrins $\alpha2\beta1$, $\alpha3\beta1$, $\alpha4\beta1$, and $\alpha5\beta1$ [2, 5, 14]. The expression level also varies depending the origin of the mast cells. Skin mast

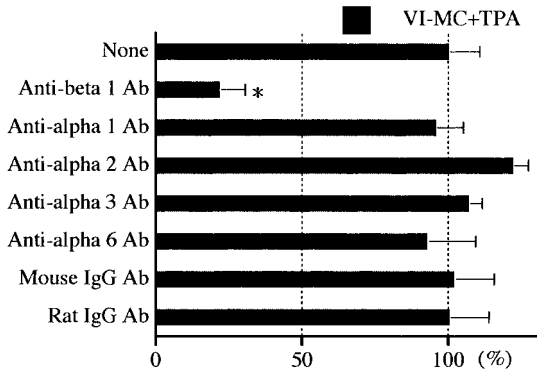

Fig. 3. Effect of anti-integrin antibodies (Abs) on the adhesion of VI-MC to laminin (LN). Cells were incubated with specific Abs or control Abs (mouse or rat IgG Ab) in the presence of phorbol ester (TPA). Results represent means ± SD of 3 experiments. * $P<0.05$ indicates a significant difference compared with the controls (None, without any Ab). The number of LN-adherent cells without Ab is defined as representing 100% adhesion.

cells express low levels of integrins $\alpha4\beta1$ and $\alpha5\beta1$ (less than 10%), while almost all mast cells from the uterus and intestines are positive for these integrins. On the other hand, human mast cell line HMC-1 expresses integrins $\beta1$ and $\alpha2$ through $\alpha6$, although their expression levels are variable [11, 21].

Integrin expression varies both between and within different tumor types. For example, human melanoma cells are reported to exhibit heterogeneity of integrin expression in many studies on cell lines [23]. In this study, all MCT cells showed similar expression patterns for the integrin subunits despite their origins. Small differences in integrin expression depending on cell origin may be a characteristic of canine MCT cells, and upregulation of integrin expression may have occurred during tumorigenesis [1, 13]. The high expression rates of integrins in canine MCT cells might be associated with acquisition of a more invasive phenotype. Further study is necessary to clarify the significance of the similar expression patterns of integrins in MCT cells.

Because expression of integrins alone does not indicate that they are functional, adhesion of MCT cells to the ECM was also assessed. Previous studies with canine MCT cells have shown that these cells poorly attach to FN, Coll I, and Coll IV without stimuli [22]. In the reports on human mast cells, the binding ability to the ECM via integrins and necessity of activation by mediators varies. Skin mast cells weakly bind to FN and LN without stimulation, and do not bind to Coll I and IV [2]. Intestinal mast cells spontaneously adhere to FN, and the degree of adherence is enhanced by stem cell factor [14]. Human mast cell line HMC-1 adheres to FN, LN, and Coll I without any stimulation [11].

In this study, the adhesive properties differed among the canine MCT cell lines, although all the cells expressed the same integrin patterns. CoMS adhered to FN spontaneously, and CM-MC and VI-MC required activation by TPA for adhesion. The fact that all cells adhered to FN indicates that FN might be an important ECM for canine MCT. All cell lines expressed integrins that might function as an LN receptor; however, VI-MC were the only cells that adhered to LN. The difference among the cell lines observed in this study might indicate the nature of the cell origin. VI-MC originated from intestinal MCTs, which have high potential for hematogenous metastasis [18]. Since LN is abundant in the basal lamina of blood vessels, the ability of VI-MC to adhere to LN may play a role in dissemination in intestinal MCTs.

No MCT cells adhered to Coll I and IV instead of the expression of Coll receptors. Although the precise cause of this is unclear, it might have been due to failure of integrins on the MCT cells to function as Coll receptors, unsuitability of the substrate origin for the action, or unsuitability of the activating stimuli. Few mast cells have been found to adhere to Coll despite many experiments [11, 21], and therefore low adhesion to Coll might be a characteristic of mast cells.

In order to induce adhesion, integrins need to be appropriately activated by the ECM or other stimuli [6, 7]. Since the CoMS cells adhered to FN without stimulation, FN itself may activate the FN receptors of CoMS; the degree of activation was increased by TPA stimulation. On the other hand, CM-MC and VI-MC cells needed to be activated by TPA for adhesion to FN. Phorbol ester is a potent activator of PKC and is known as a modulator of integrin-mediated adhesion. In mouse bone marrow-derived mast cells, one of the pathways that activates adhesion to FN via integrin $\alpha5\beta1$ is initiated by phospholipase C-$\gamma1$, which is dependent on PKC [10]. Although this is data related to normal mast cells, a similar mechanism might exist in canine MCT cells. Another important pathway that upregulates integrin activity is via phosphatidylinositol 3-kinase activation, which is upregulated by FcεRI, c-kit, or platelet-derived growth factor receptor [10]. Kruger-Krasagakes *et al.* suggested that mast cells were far more active in their natural tissue environment than is suggested from *in vitro* data, since additional activation of mast cells that had adhered to the ECM by secretory stimuli causes significantly enhanced cytokine gene expression and secretion [12]. Mast cells may only react in an innate manner *in vitro* after activation, and this may imply a slight difference in terms of whether stimulation is required for adherence to the ECM *in vitro*.

Our results indicated that integrin $\alpha5\beta1$ mediated adhesion to FN in the MCT cell, and that the degree of mediation differed among the cells. Anti-integrin $\alpha5$ Ab almost completely inhibited adhesion to FN in the CoMS cells, and anti-integrin $\beta1$ Ab highly inhibited it in the CM-MC and CoMS cells. Integrin $\alpha5\beta1$ plays an important role in CM-MC and CoMS cells, as shown in human intestinal mast cells and HMC-1 [11, 14]. On the other hand, VI-MC cell adhesion to FN was moderately inhibited by anti-integrin $\alpha5$ and $\beta1$ Ab by up to 50%. It has been reported that the integrin $\alpha v\beta3$ on the surface of human mast cells can mediate cell adhesion to FN [16]. Other non-$\beta1$ integrins, such as integrin

αvβ6, α4β7, and αvβ8, might mediate adhesion to FN [7]. Adhesion to FN has been found to be RGD-dependent in human and mouse mast cells [3, 11]. Although all the MCT cells expressed integrin α5β1 as a functional receptor to FN, the role of the RGD sequence on FN is still obscure. Other sequences, such as the PHSRN peptide, might be associated with adhesion to FN in these cells [4].

Anti-integrin β1 Ab inhibited adhesion of VI-MC to LN, but the anti-integrin α Abs had no effect. The integrin β1 subunit plays, at least in part, an important role in VI-MC cell adherence to LN. However, as with adhesion to FN, anti-integrin β1 Ab could not completely inhibit adhesion to LN. Mouse mucosal mast cell homologues have been proven to express the integrin α7 subunit as an LN receptor [15], and this subunit might be associated with VI-MC cell; however, we could not evaluate integrin α7 in this study due to inavailability of blocking Ab. Other non-β1 integrins, such as integrin α6β4 and αvβ8, might also mediate adhesion to LN [7].

β1 integrins also play a critical role in initiation and maintenance of tumor growth [1, 13]. Further investigation using canine MCT tissues and comparison with normal canine mast cells is required to clarify the role of the integrin β1 subfamily in canine MCTs. In human medicine, integrin α5β1 is drawing attention as a potent modulator of mast cell function, and it may be utilized in future therapies against allergies [6]. Studies of integrin expression and function are increasing our understanding of the cell interactions of canine MCTs and may pave the way for new prognostic tools and novel therapeutic interventions.

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