Brazilian Journal of Medical and Biological Research (2003) 36: 1101-1109
ISSN 0100-879X

Differential expression of integrin subunits on adherent and nonadherent mast cells

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Abstract

Mast cell progenitors arise in bone marrow and then migrate to peripheral tissues where they mature. It is presumed that integrin receptors are involved in their migration and homing. In the present study, the expression of various integrin subunits was investigated in three systems of adherent and nonadherent mast cells. Mesentery mast cells, freshly isolated bone marrow-derived mast cells (BMMC) and RBL-2H3 cells grown attached to tissue culture flasks are all adherent mast cells and peritoneal mast cells, and cultured BMMC and RBL-2H3 cells grown in suspension represent nonadherent mast cell populations. Pure populations of mast cells were immunomagnetically isolated from bone marrow, mesentery and peritoneal lavage using the mast cell-specific monoclonal antibody AA4. By immunomicroscopy, we could demonstrate that all of these mast cells expressed α4, α5, α6, ß1 and ß7 integrin subunits. The expression of the α4 integrin subunit was 25% higher in freshly isolated mesentery mast cells and BMMC. Consistent with the results obtained by immunomicroscopy, mesentery mast cells expressed 65% more mRNA for the α4 integrin subunit than peritoneal mast cells. *In vitro* studies were also conducted using the rat mast cell line RBL-2H3. RBL-2H3 cells grown attached to the tissue culture flasks or as suspension cultures expressed the same integrin subunits identified in bone marrow, mesenteric and peritoneal mast cells *ex vivo*. Similarly, the expression of α4 integrin was higher in adherent cells. Therefore, α4 integrins may play a critical role in the anchorage of mast cells to the extracellular matrix in bone marrow and in peripheral tissues.

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Presented at SIMEC 2002 (International Symposium on Extracellular Matrix), Angra dos Reis, RJ, Brazil, October 7-10, 2002.

Research supported by FAPESP (Nos. 99/11133-8, 99/07823-3, and 99/11137-8). A.C.G. Grodzki and J.F. Sousa are recipients of FAPESP fellowships. M.V. Dávila Pástor is the recipient of a UNIVALI fellowship.

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Received November 14, 2002
Accepted July 8, 2003

Key words
• Mast cells
• Integrin
• Mouse

Introduction

Mast cells are important immunoregulatory and inflammatory cells preferentially found in perivascular connective tissues of multiple organs. Committed mast cell precursors derived from bone marrow migrate to peripheral tissues where they complete their maturation into phenotypically distinct mast cells (1-4). The mechanism underlying differentiation, migration and tissue-specific homing of mast cell precursors is not completely understood. It is presumed that the interaction of specific cell adhesion receptors expressed on the surface of mast cells with extracellular matrix proteins plays an

important role (5-7). Integrins are αß heterodimeric transmembrane receptors widely expressed on migratory cells, that mediate cell-extracellular matrix adhesion (8) and are involved in signal transduction pathways modulating diverse cellular processes such as cell migration, differentiation and proliferation (9-12). *In vitro* studies have shown that cultured mast cells express integrin receptors for extracellular matrix proteins such as fibronectin, vitronectin and laminin (13-17). However, studies of mast cell adhesion *in situ* have been limited by lack of mast cell-specific markers, since mast cells in early stages of maturation cannot be differentiated from other cell types on the basis of their morphology alone. Using the monoclonal antibody (mAb) AA4 (18) that recognizes two derivatives of the ganglioside G_{D1b} (19), which are unique to the surface of rodent mast cells (20), pure populations of mast cells can be isolated (21).

In the present study, we investigated the expression of α4, α5, α6, ß1 and ß7 integrin subunits on adherent and nonadherent populations of mast cells isolated from mouse bone marrow, rat mesentery and rat peritoneal lavage and RBL-2H3 cells, a mast cell line. Freshly isolated bone marrow and mesentery mast cells are found, *in vivo*, in association with extracellular matrix in connective tissue. In contrast, cultured bone marrow-derived mast cells (BMMC) grow in suspension and mast cells from the peritoneal lavage are considered to be free cells found in the abdominal cavity and therefore studied as nonadherent cells. RBL-2H3 cells could be grown attached to tissue culture flasks or in suspension in spinner flasks as nonadherent cells. We compared the expression of various integrin subunits on adherent and nonadherent mast cells in order to investigate the probable role of specific integrin molecules in rodent mast cell migration and homing. In addition, we compared the expression of the same integrin subunits in freshly isolated bone marrow and cultured BMMC in order to assess the modulation of integrin expression during mast cell maturation.

Material and Methods

Cells

Young (8-12 weeks) male BALB/c mice and young (150 g) male Wistar rats from the animal breeding facilities of FMRP-USP, Ribeirão Preto, SP, Brazil, were used. Animals were housed and experiments were conducted according to institutional protocols and guidelines. Bone marrow was removed from the mouse femurs with Iscove's medium (Life Technologies/Gibco, Rockville, MD, USA) containing 1000 U/ml heparin (Produtos Roche Químicos e Farmacêuticos, Rio de Janeiro, RJ, Brazil) and 1000 U/ml DNase type I (Sigma, St. Louis, MO, USA). The cells were dissociated by repeated aspiration with a Pasteur pipette and then rinsed twice by centrifugation at 27 *g* in Iscove medium containing 2% BSA.

Cells were dissociated from rat mesentery by incubating mesentery fragments with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Life Technologies/Gibco) containing 0.76 mg/ml ethylenediaminetetraacetic acid (EDTA; Life Technologies/Gibco) for 15 min, followed by incubation in Iscove's medium containing 1.25 mg/ml collagenase (Worthington Biochemical Co., Lakewood, NJ, USA) and 150 U/ml hyaluronidase (Sigma) for 1 h, in a spinner flask, at 37ºC.

Peritoneal cells were obtained by injecting rats *ip* with 20 ml sterile PBS. The peritoneal lavage was collected with a Pasteur pipette after laparotomy.

The rat mast cell line, RBL-2H3, was grown either as adherent monolayers in tissue culture flasks as described previously (22) or in suspension culture in spinner flasks.

Antibodies

mAb AA4, a generous gift from Dr.

Reuben Siraganian (NIDCR, NIH, Bethesda, MD, USA), was raised in mice against the cell surface of RBL-2H3 cells (18). Antibodies that recognize rat or mouse α4, α5, α6, ß1 and ß7 integrin subunits were purchased from Santa Cruz Biotechnologies (anti-rat; Santa Cruz, CA, USA) or Pharmingen (anti-mouse; San Diego, CA, USA).

Coupling of antibodies to magnetic beads

mAb AA4 was conjugated to tosylactivated Dynabeads (Dynal Biotech, Lake Success, NY, USA) as previously described (23). Briefly, equal volumes of tosylactivated Dynabeads at 4 x 10^8 beads/ml and mAb AA4 at 300 µg/ml were mixed. The solution was incubated for 24 h at 22ºC with slow end-over-end rotation. After incubation the magnetic beads were washed three times with 10 mM PBS containing 0.1% BSA in a magnetic particle concentrator (Dynal Biotech). The coated beads were resuspended and stored in PBS + 0.1% BSA at a concentration of 4 x 10^8 beads/ml.

Cell separation

The suspension of bone marrow cells, mesentery cells, or peritoneal lavage was washed twice by centrifugation at 27 *g* in Iscove's medium containing 2% BSA and 5 µg/ml normal mouse IgG. The cell suspension was first incubated for 30 min at room temperature with IgG-coated beads in PBS containing 2% BSA. The cell suspension was then incubated with mAb AA4-coated beads (3 beads/target cell) in PBS containing 2% BSA for 10 min at 16ºC. After incubation, mast cells were isolated by washing twice in PBS containing 2% BSA and twice in PBS, using the magnetic particle concentrator.

Immunolabeling

mAb AA4-isolated mast cells were rinsed twice in PBS, and placed on Cell Tak (BD Biosciences, Bedford, MA, USA)-coated coverslips, washed again in PBS, fixed with 2% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized with -20ºC acetone for 5 min. The cells were rinsed in PBS and then in PBS containing 0.1 M glycine, blocked with 1% BSA in PBS + 5 µg/ml of donkey IgG and finally incubated with antibodies against α4, α5, α6, ß1 or ß7 integrin subunits (20 µg/ml; Pharmingen). After incubation with primary antibodies, cells were rinsed thoroughly in PBS, incubated with donkey anti-goat $F(ab')_2$ conjugated to FITC (Jackson ImmunoResearch, West Grove, PA, USA) or donkey anti-rat $F(ab')_2$ conjugated to FITC (Jackson Immuno-Research). All cells were rinsed in PBS and then briefly in distilled water and the coverslips were mounted with Fluoromount G (EM Sciences, Fort Washington, PA, USA).

Fluorescence microscopy

The mast cell images were acquired by scanning confocal microscopy (Leica TCS NT, Heidelberg, Germany). The confocal parameters were established at the beginning of the study and remained constant throughout, with an equal brightness setting and a confocal aperture pinhole setting of 1. The images were visualized with a 100X oil immersion objective.

A standard slide for green fluorescence Fluor-Ref™ (Fluorescence Reference Slides, Microscopy Education, www.Microscopy Education.com) was used to standardize the laser output and Photo Multiplier settings for the confocal microscope, in order to compare images acquired from different experiments.

Image analysis

A total of 608 immunolabeled mast cells were evaluated by image analysis and by measuring the ratio of green fluorescence intensity per area (μ^2) using the Image Pro-

Plus program (Media Cybernetics, version 4.0).

Cell culture

After isolation, mAb AA4+ mouse BMMC were cultured in T-25 flasks (Costar 3056), 1 x 10^5 cells/ml, in Iscove's medium supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), an antimycotic agent (0.25 µg/ml amphotericin B; Life Technologies), 50 µM ß-mercaptoethanol, 10% fetal calf serum, 100 ng/ml recombinant stem cell factor (Biosource International, Nivelles, Belgium) and 20 ng/ml recombinant IL-3 (Biosource International). Complete medium (0.5 ml) was added to each flask every 5 days. Isolated cells were not removed from the magnetic beads prior to being placed in culture.

The RBL-2H3 cells were maintained in Dulbecco's minimum essential medium supplemented with 15% fetal calf serum, penicillin and streptomycin as previously described (22,24).

RT-PCR

Total RNA was extracted from rat mesentery and peritoneal mast cells that had been isolated with mAb AA4. The extracted RNA was treated with DNase (Promega, Madison, WI, USA) to eliminate residual genomic DNA. cDNA was synthesized with SUPERSCRIPT II Reverse Transcriptase (Life Technologies) using an oligo $(dT)_{12-18}$ primer (Life Technologies) and random hexamer primers (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Four pairs of oligonucleotide primers (Invitrogen, São Paulo, SP, Brazil) were used for amplification for 45 s at 45ºC, 1 min at 55ºC, and 1 min at 72ºC for 32 cycles (ß7 integrin primers), 34 cycles (α4 and ß1 integrin primers) or 36 cycles (α2 integrin primers), using Taq DNA Polymerase (Amersham Pharmacia Biotech). Primers for ß-actin, a housekeeping gene, were used as an endogenous control. The PCR products were analyzed on 2.0% agarose gels.

Primers

The following primers were used: integrin α2: 5' TGACCGGGATACAAACAG ACA 3' (sense), 5' CATGAGGGGAATCG TGACAG 3' (antisense); integrin α4: 5' AAAGGCAGTACAAATCTATCC 3' (sense), 5' GAGCCCACCTAATCAGTAAT 3' (antisense); integrin ß1: 5' GGGCCAACCT GTGAGACCT 3' (sense), 5' GCCCCAAAG CTACCCTACTGT 3' (antisense); integrin ß7: 5' ACCGGCTCTCAGTGGAAGTCT 3' (sense), 5' TACAGCACAGGCCGAAAG TCT 3' (antisense); ß-actin: 5' CTAAGGCCA ACCGTCAAAAGA 3' (sense), 5' ATTGCC GATAGTGATGACCTG 3' (antisense).

Flow cytometry

RBL-2H3 cells grown in suspension or as adherent monolayers were immunolabeled with antibodies against the α4, α5, α6 and ß7 integrin subunits and analyzed by flow cytometry. RBL-2H3 cells grown as an adherent monolayer were harvested using trypsin/0.53 mM EDTA. Both the adherent and non-adherent RBL-2H3 cells were washed twice in PBS by centrifugation, permeabilized with acetone at -20ºC, for 1 min, rinsed in PBS, blocked for 30 min in 1% BSA and then in PBS containing 5 µg/ml donkey IgG, and incubated with the same antibodies against the integrin subunits used for peritoneal and mesentery mast cells. After incubation, cells were washed in PBS, incubated with donkey anti-goat IgG $F(ab')_2$ conjugated with FITC, washed three times in PBS and analyzed with a Fluorescence Activated Cell Sorter (FACSort; BD Biosciences).

Membrane fractions

Freshly isolated and cultured BMMC were

washed and resuspended in cold phosphate buffer containing 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol (DTT; Pierce Chemical Co., Rockford, IL, USA), and the protease inhibitors 20 µg/ml aprotinin (Sigma) and 10 µg/ml phenylmethylsulfonyl fluoride (Sigma). The cells were disrupted with a Kontes Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ, USA) on ice and then centrifuged at 200 *g* for 10 min. The supernatant was then centrifuged at 45,500 *g* for 20 min. The pellet was resuspended in 10 mM Tris, pH 7.6, containing 1 mM EDTA and 1 mM DTT, and centrifuged at 126,000 *g* for 1 h at 6ºC on a discontinuous sucrose gradient (25 and 42%). The membrane fraction was collected from the interface between the 25% and 42% sucrose fractions and diluted in 10 mM Tris, pH 7.6, containing 1 mM EDTA and 1 mM DTT and centrifuged at 208,000 *g* for 1 h at 6ºC. The membrane fraction was analyzed by immunofluorescence microscopy using an antibody against the ß1 integrin subunit and by electron microscopy. The pellet was frozen at -70ºC and used for enzyme-linked immunosorbent assays (ELISA).

ELISA

Ninety-six-well plates were coated with the mast cell membrane fraction (10^2 cells/well) in 0.2 M sodium carbonate buffer, pH 9.6, overnight at 4ºC. The plates were washed with PBS + 0.05% Tween 20 (Tween 20R; Sigma), blocked with PBS containing 0.05% Tween 20 and 5 µg/ml donkey IgG, and incubated with the antibodies against the integrin subunits. The primary antibodies used for ELISA were the same as those used for immunofluorescence, but at a lower concentration (5 µg/ml). After incubation with primary antibodies, the membrane fractions were washed in PBS + 0.05% Tween 20, incubated with donkey anti-rat IgG $F(ab')_2$ conjugated with horseradish peroxidase (Jackson ImmunoResearch) for 30 min and washed thoroughly in PBS. The ELISA FEMTO chemiluminescent kit (Pierce) was used for detection and a Microplate Luminometer Series 7700, version 4.03 (Bio-Tech Industries, Winooski, VT, USA) was used to read the assays.

Results

Integrin subunit expression was analyzed by immunological methods on adherent and nonadherent populations of mast cells isolated from mesentery, peritoneal lavage, and bone marrow and on the mast cell line, RBL-2H3. Mast cells from different tissues were used to compare the integrin expression on cells found associated with extracellular matrix, such as mesentery and bone marrow mast cells, with the expression on nonadherent mast cells, such as peritoneal mast cells and cultured BMMC in order to investigate the probable role of specific integrin subunits in rodent mast cell migration and homing. *In vitro* studies were also conducted using the rat mast cell line RBL-2H3 grown attached to tissue culture flasks or as suspension cultures. The comparison of both of these conditions, adherent and nonadherent, can be useful in investigating the expression of adhesion molecules that are related to recruitment and tissue-specific homing, such as the detachment of mast cells from the bone marrow stroma in order to migrate to peripheral tissues.

Differential expression of integrin subunits on mast cells

By immunofluorescence, both freshly isolated mesentery mast cells and peritoneal mast cells express the same integrin subunits, α4, α5, α6, ß1 and ß7. However, the expression of the α4 integrin subunit appeared to be higher in the mesentery mast cells (Figure 1).

Both freshly isolated and cultured BMMC populations expressed α4, α5, α6, ß1 and

ß7 integrin subunits on their surface (Figure 2). However, the expression of the ß1 integrin appeared to be higher than that of the other subunits (Figure 2C,F). The freshly isolated mast cells from bone marrow also showed a higher expression of α4 integrin subunit (Figure 2A). According to the analysis of fluorescence intensity, the expression of α6, ß1 and ß7 integrin subunits increased while the expression of α4 and α5 integrin subunits decreased after 25 days in culture (Figure 3A). Quantitative analyses were also carried out by ELISA to compare the expression of the integrin subunits on freshly isolated and cultured BMMC (Figure 3B). It was possible to detect α4, α5, α6, ß1 and ß7 integrin subunits on freshly isolated BMMC and it was also observed that the fluorescence intensity of all the integrin subunits increased with BMMC maturation. Therefore, our data suggest that freshly isolated and cultured BMMC express the same integrin subunits, but the level of expression of each subunit changes with cell maturation *in vitro*.

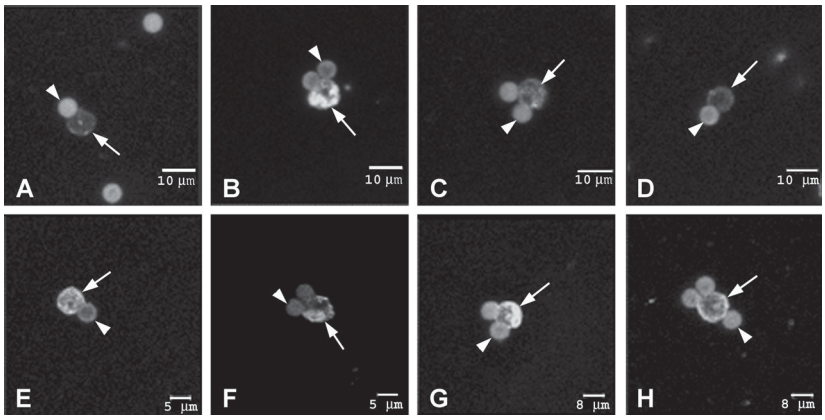

Figure 1. Expression of α2 (A,E), α4 (B,F), α5 (C,G) and α6 (D,H) integrin subunits in mast cells (arrows) immunomagnetically isolated from the rat mesentery (A-D) and in mast cells (arrows) immunomagnetically isolated from the rat peritoneal lavage (E-F). Immunomagnetic beads are indicated by the arrowheads.

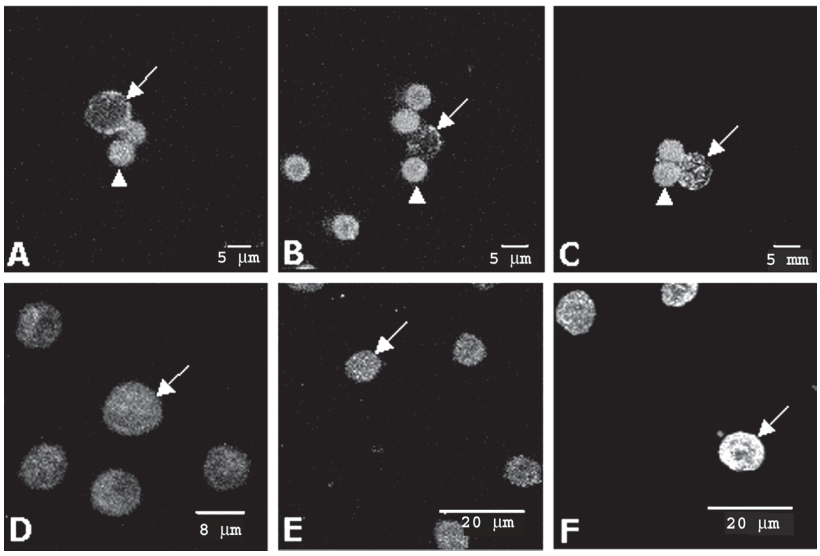

Figure 2. Freshly isolated bone marrow-derived mast cells (BMMC; A,B,C; arrows) express the integrin subunits α4 (A), α6 (B) and ß1 (C). After 25 days in culture BMMC (D,E,F; arrows) also expressed the integrin subunits α4 (D), α6 (E) and ß1 (F). Immunomagnetic beads are indicated by the arrowheads.

High expression of α4 on adherent mesentery mast cells

To further investigate the differential expression of the α2, α4, ß1 and ß7 integrin subunits, the presence of mRNA for these subunits was evaluated by semiquantitative RT-PCR. The RT-PCR results showed that both mesentery mast cells and peritoneal mast cells express mRNA for the α2, α4, ß1 and ß7 integrin subunits. Confirming the immunofluorescence results, the expression of mRNA for the α4 integrin subunit was 65% higher in mesentery mast cells than in peritoneal mast cells (Figure 4), and the peritoneal mast cells expressed 97% more mRNA for the α2 integrin subunit.

Differential expression of integrin subunits on RBL-2H3

In order to confirm the differences observed between the expression of the integrin subunits in adherent mesentery and freshly isolated BMMC and nonadherent peritoneal mast cells and cultured BMMC, RBL-2H3 cells were used for further *in vitro* studies. RBL-2H3 cells grown as adherent monolayers or grown in suspension were immunolabeled with antibodies against the α4, α5, α6 and ß7 integrin subunits and analyzed by flow cytometry. RBL-2H3 cells grown either

attached or in suspension expressed the same integrin subunits, α2, α4, α5, α6, ß1 and ß7, detected by immunofluorescence in adherent and nonadherent mast cells. The expression of the α4 integrin subunit was also higher in adherent RBL-2H3 cells than in cells grown in suspension (Figure 5).

Discussion

The present study provides evidence that adherent mast cells express higher levels of α4 integrin than nonadherent mast cells. The expression of the α4 integrin subunit was higher in adherent mast cells: mesentery mast cells, freshly isolated BMMC and adherent RBL-2H3 mast cells. The α4 integrin subunit has been related to the proliferation and differentiation of progenitor cells from bone marrow (25) and has been shown to participate in the migration process by interactions with vascular cell adhesion molecule-1 (26). The higher expression of the α4 subunit in all the adherent mast cells analyzed may be related to an increase in the expression of the fibronectin receptors (α4ß1, α4ß7). Previous studies have suggested that α4ß7 integrin is required for mast cell tissue-specific homing (27) and mast cell recruitment (28). Therefore, fibronectin receptors (α4ß1 and α4ß7) may play a critical role in anchorage and homing of mast cells attached to the extracellular matrix.

The freshly isolated and the cultured BMMC express the same integrin subunits, but the levels of expression changes with maturation *in vitro*. Recent studies have suggested that the modulation of adhesion molecules on the surface of mast cells could occur simultaneously with mast cell matura-

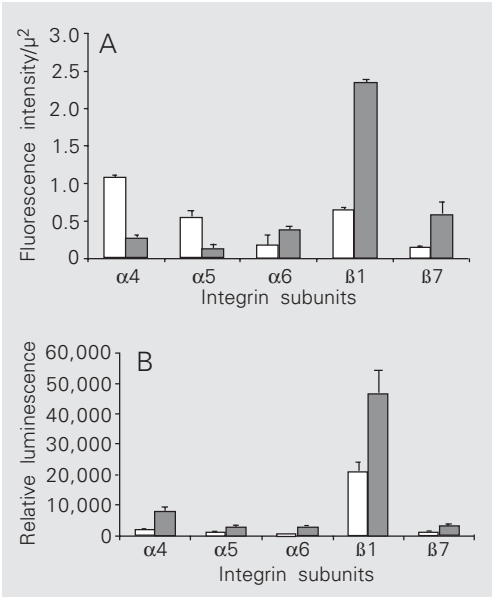

Figure 3. Expression of integrin subunits quantified by fluorescence microscopy (A) and ELISA (B). There was a higher expression of α6, ß1 and ß7 integrin subunits after 25 days in culture (A). The expression of all integrin subunits was significantly higher in the cultured bone marrow-derived mast cells (BMMC) analyzed by ELISA (B). The data are reported as means ± SEM. The differences in integrin expression between the freshly isolated (white columns) and 25-day cultures of BMMC (gray columns) were all statistically significant ($P \leq 0.05$, ANOVA).

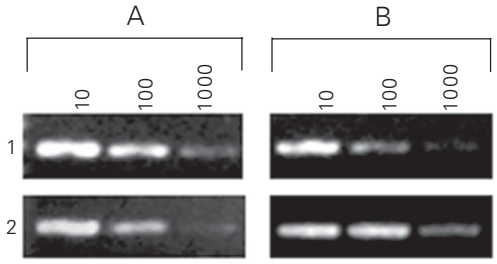

Figure 4. Semiquantitative RT-PCR analysis of the α4 integrin subunit (1) shows higher levels of mRNA expression in mesentery mast cells (A) than in peritoneal lavage mast cells (B). ß-actin (2) was used as control. The cDNA prepared from 1 µg of total RNA was diluted serially 10X, 100X and 1000X. The products were analyzed on 2% agarose gel.

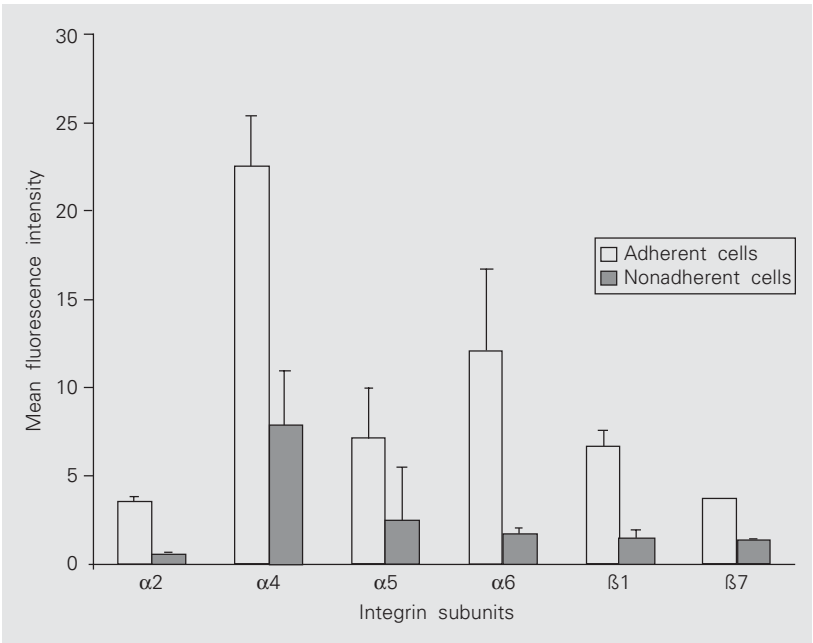

Figure 5. The expression of integrin subunits in RBL-2H3 cells grown either attached to tissue culture flasks (adherent cells) or in suspension (nonadherent cells). The integrin subunits were quantified by flow cytometry and expression was higher for all integrin subunits in adherent cells. Data are reported as mean of fluorescence intensity ± SEM.

tion and that the differential expression of these molecules allows the migration and specific homing (7,29). *In vivo*, the cells can transit through different microenvironments and are exposed to different factors during the migration and maturation processes. *In vitro* studies intend to reproduce *in vivo* conditions, but are hampered by multiple factors such as the fact that cells are continuously exposed to the same factors, mainly stem cell factor. The stimulus of the stem cell factor was shown to augment the gut mast cell adhesion and human immature mast cell adhesion (30,31). Previous studies have suggested that the interaction of stem cell factor and its receptor c-kit can alter the integrin functions and that both stem cell factor and integrins can work together to modulate the adhesion and localization of mast cells (32).

The results of the expression of α4 and α5 integrin subunits obtained by image analyses and ELISA were contradictory. This difference could be attributed to the experimental procedures used for each method. For image analyses, the cells are evaluated *ex vivo* after fixation and permeabilization and the immunolabel could represent the integrin subunit expression on the cell surface only. The access of the antibody to the transmembrane and cytoplasmic epitopes may be limited by the efficiency of permeabilization. With ELISA, the cells were mechanically lysed and the isolated membrane fraction was used. The process of cell lysis could expose more integrin epitopes than are accessible by immunofluorescence. In addition, the membrane preparations lack cytoplasmic components that may limit the access of the antibodies to their epitopes. Therefore, the ELISA procedures can show binding sites that normally are located inside the cell membrane; however, this method may not reflect the function of integrin molecules in the whole intact cell.

The high level of expression of the ß1 subunit on cultured BMMC could be also related to its heterodimerization with different α subunits. The expression of αß1 complexes is regulated by the presence of the specific α subunit since the ß1 subunit is constantly present in excess (33). The heterodimerization of the ß1 subunit with different α subunits may improve the BMMC attachment to different components of the extracellular matrix and may regulate the migration of immature mast cells. The ability of BMMC to migrate to peripheral tissues is determined in part by their integrin receptors as well as by the microenvironment surrounding the cells. Therefore, the modulation of integrins on the surface of BMMC could play an important role in mast cell migration from the bone marrow to peripheral sites.

Acknowledgments

We wish to thank Márcia Sirlene Zardin Graeff, Department of Cell and Molecular Biology and Pathogenic Bioagents, for technical assistance with the confocal microscope, Alexandra Rosa Vieira Dias, Department of Biochemistry and Immunology, for assistance with the FACS analysis, and Dr. Norberto Garcia-Cairasco, Department of Physiology, FMRP-USP, for making available the Image Pro Plus 4.0 software.

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