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Heterotypic cell-cell adhesion of human mast cells to fibroblasts

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Abstract **Skin mast cells are typically located in the perivascular or perineural connective tissue. We observed that HMC-1 mast cells growing in suspension adhered efficiently to (> 90% of cells) and spread on top of fibroblast monolayers and to a lesser degree on purified extracellular matrix proteins. Since adhesive interactions determine cell migration and tissue localization we studied the mechanism. It was found that HMC-1 cells attach to collagen I and fibronectin, laminin, collagen IV and vitronectin, but not to collagens III and VI or hyaluronic acid. Adhesion to fibronectin, collagen I and laminin was completely inhibited by mAbs blocking β_1-integrins, whereas adhesion of HMC-1 cells to vitronectin was inhibited by anti-α_v-chain mAbs. However, attachment of HMC-1 cells to fibroblasts was not influenced by mAbs blocking β_1- or α_v-chain function, by RGD peptides or by mAbs interfering with other receptors, most notably c-*kit*. Identical results were obtained with normal mast cells isolated from human foreskin. These results indicate that human mast cells attach to fibroblasts independently of β_1- or α_v-integrins as well as of c-*kit* receptor-mediated mechanisms. The functional characteristics observed (i.e. only partial sensitivity to trypsin and EDTA, no increase in trypsin sensitivity by pretreatment with EDTA) suggest that cadherin receptors were not involved, and it is likely that the adhesion process observed involved not-yet-defined heterotypic cell-cell adhesion receptors.**

Key words HMC-1 cell line · Extracellular matrix protein · Integrin · c-*kit* · Cadherin

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Introduction

Mature mast cells are preferentially located in the subepithelial connective tissue of the skin, gastrointestinal tract and lung. They play a central role in inflammatory and allergic reactions, and are also involved in tissue remodelling during wound healing [1]. Mast cells of various species have been found to interact with different cell types, including fibroblasts, vascular endothelial cells, epithelial cells, lymphocytes, macrophages, neutrophils, eosinophils, nerve cells and cancer cells [2–12]. Among the best studied are murine mast cells which are known to proliferate and differentiate in vitro after attachment on top of fibroblast monolayers [13, 14]. However, the adhesive mechanisms, initiating and/or maintaining the underlying cell-cell interactions are not well understood.

The present study was designed to identify the cellular and extracellular components of the connective tissue to which human mast cells adhere and to characterize the receptors involved. We found that human mast cells specifically adhere to a number of extracellular matrix (ECM) compounds, e.g. collagens (Coll), fibronectin (FN), laminin (LM) and vitronectin (VN). Adhesion to these ECM components was exclusively mediated by β_1- and α_v-integrins. Moreover, HMC-1 cells not only adhered to macromolecules of the ECM produced by fibroblasts but also attached on top of confluent fibroblast monolayers with high efficiency. Interestingly, the latter adhesion process was independent of β_1- and α_v-integrins, indicating that other receptors probably mediating heterotypic cell-cell attachment are operating. Adhesion assays employing normal mast cells isolated from human foreskin demonstrated that the observed heterotypic cell-cell interaction not only applies for a human mast cell line but also for the normal untransformed counterpart. Our results suggest that human mast cells utilize yet unidentified receptors for adhesion to fibroblasts.

Materials and methods

Cell lines and culture conditions

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, 1% nonessential aminoacids, 100 U/ml penicillin and 100 U/ml streptomycin (all purchased from Linaris, Bettingen, Germany) at 37° C in an atmosphere containing 5% CO_2.

The suspension growing human mast cell leukemia line HMC-1 was maintained as previously described [15]. The fetal fibroblast cell line F135-60-86-skin was originally obtained from Dr. J. Fogh's cell bank at the Memorial Sloan Kettering Cancer Center, New York, N. Y. The BALBc/3T3 fibroblast cell line was provided by M. Schupp (Department of Virology, University Würzburg, Germany). All cell lines were repeatedly subjected to hybridization tests using ^{3}H-labelled mycoplasma DNA (Mycoplasma TC Gene Probe, San Diego, Calif.). All the tests were negative.

Preparation of human mast cells

Suspensions of human skin mast cells were prepared by enzymatic digestion of human foreskin obtained after circumcision. Skin was immediately placed in HEPES-buffer (137 m*M* NaCl, 5.6 m*M* D-glucose, 10 m*M* HEPES, 2.7 m*M* KCl, 0.4 m*M* NaH_2PO_4, 0.5 m*M* $MgCl_2$, 1.0 m*M* $CaCl_2$; pH 7.3), stored at 4° C and processed within 1 h. The skin was cut with scissors, and the tissue fragments were washed twice in HEPES-buffer containing 0.1% bovine serum albumin (HEPES-BSA). After incubation in HEPES-BSA containing 1.5 mg/ml collagenase and 0.75 mg/ml hyaluronidase for 2 h at 37° C in a shaking water bath, the resulting cell suspension was filtered through gauze and undigested tissue was incubated with fresh enzyme for a second digestion period. Dispersed cells were washed twice in HEPES-BSA, recovered by centrifugation and stored at 4° C in PIPES-A (137 m*M* NaCl, 5.6 m*M* D-glucose, 10 m*M* PIPES, 2.7 m*M* KCl, 0.1% human serum albumin; pH 7.4) before use.

Monoclonal antibodies

The antibodies employed for assessing expression of cell surface adhesion and other receptors on the human mast cell line HMC-1 as well as human skin fibroblasts are listed in Table 1. Serum and ascites of hybridoma-bearing mice or tissue culture supernatant was used. Isotype-matched monoclonal antibodies (mAbs) recognizing cell surface antigens not expressed on HMC-1 cells and fibroblasts were used as controls. For inhibition studies, function-blocking mAbs were purified by ammonium sulphate precipitation followed by affinity chromatography using standard procedures.

Flow cytometry analysis

Cell surface expression of the receptors was analysed by flow cytometry. Briefly, cells (5×10^5) were washed with phosphate-buffered saline (PBS) + 1% BSA, incubated with primary antibodies for 30 min, washed again three times and then incubated with FITC-labelled $F(ab')_2$ fragments of rabbit anti-mouse or rabbit anti-rat Ig (Dako, Glostrup, Denmark) for 30 min. After three final washes, resuspended cells were analysed using a FACScan (Becton Dickinson, Mountain View, Calif.)

Radioimmunoprecipitation

Immunoprecipitation was carried out as previously described [30]. Briefly, samples of NP-40-solubilized cell extracts were incubated with mAbs for 1.5 h at 4° C. Immune complexes were then precipitated with protein A Sepharose (Pharmacia, Uppsala, Sweden) precoated with rabbit anti-mouse Ig (Dako). After repeated washes (0.5% NP –40, 0.1% SDS, 0.15 *M* NaCl, 0.01 *M* Tris; pH 7.5) the precipitated proteins were eluted with SDS-PAGE sample buffer and processed by SDS-PAGE on 9% polyacrylamide gels under reducing and nonreducing conditions. For fluorography, gels were immersed in 0.5% sodium salicylates for 20 min, dried and exposed to X-ray film (Kodak X-omat).

Adhesion assays

Assays were carried out as previously described [31]. Briefly, cells were labelled with the fluorescent dye PKH2-GL according to the protocol of the manufacturer (Sigma, St. Louis, Mo.).

To determine cell adhesion to ECM macromolecules, 96-well flat bottomed plates were coated with Coll I, III (Biomol, Hamburg, Germany) IV or VI, FN, LM, hyaluronic acid (HA) (all from Sigma), VN (Biomol), or BSA (Sigma) at 4° C for 16 h. Nonspecific binding sites were blocked by subsequent incubation with 1% BSA in RPMI-1640 for 30 min. To determine cell adhesion to the ECM proteins laid down by cultured fibroblasts, F135-60-86 skin cells were cultured for 72 h in 96-well flat bottomed plates. After removing the cells with NP-40 buffer (0.5% NP-40, 2 m*M* phenylmethylsulphonylfluoride, 4 μg/ml aprotinin; all from Sigma) and three washes with PBS, the nonspecific binding sites were blocked as described above. Labelled cells (2.5×10^5/well, viability ≥ 95%) were then seeded into the coated 96-well flat bottomed plates and incubated for 30 min at 37° C. To remove nonadherent cells, wells were carefully washed three times with PBS followed by gentle swirling, inversion of the plate and blotting of excess liquid onto filter paper. Fluorescence of bound cells was measured using a Fluoroscan II fluorescence plate reader (Flow Laboratories, Meckenheim, Germany) using 485 nm excitation and 583 nm emission filters which corresponded well with the excitation and emission maxima of the fluorescence dye.

The percentage of attached cells was determined by comparison with a standard curve. For standardization of cell fluorescence twofold serial dilutions of stained HMC-1 cells were prepared ranging from 1×10^6 to 1.95×10^3 cells/well. Fluorescence intensity was directly correlated with the number of labelled cells. The threshold of detectability was 1×10^4 cells/well (≈ 4% of loaded cells).

As models of heterotypic cell-cell adhesion of mast cells, the human skin-derived fibroblast cell line F135-60-86 skin and the mouse embryonic cell line BALBc/3T3 were used. Fibroblasts (4×10^4 cells/well) were plated in 96-well flat bottomed plates and maintained at 37° C in an atmosphere containing 5% CO_2 for at least 18–24 h. After reaching confluence fibroblast monolayers were employed in adhesion assays. To avoid nonspecific binding, the monolayers were preincubated with 1% BSA in RPMI-1640 for 30 min.

The adhesion of normal mast cells isolated from human skin was assayed using an enzyme-linked immunosorbent assay (ELISA) to determine the total histamine content after lysis of the attached cells (according to the protocol of the manufacturer, Dianova, Hamburg, Germany). Adhesion assays were done in triplicate. For comparing data, mean values and standard deviations of cell numbers were calculated.

Inhibition assays

To study the functional contribution of receptors for HMC-1 adhesion, the following function blocking mAbs were introduced into the adhesion assays (for specificities, see Table 1): Integrin β_1-chain, 4B4, mAb13; α_1-chain, 1B3.1; α_2-chain, 5E8, 6F1; α_3-chain, P1B5; α_4-chain, P4G9; α_5-chain, P1D6; α_6-chain, GOH3; α_v-chain, 17E6; $\alpha_v\beta_3$-integrin, LM609; $\alpha_v\beta_5$-integrin, P1F6; and $\alpha_E\beta_7$-integrin, 28Cl2, 26F1, 2G9. Other receptors included: LFA-1, 25.3.1; LFA-3, AICD58; E-cadherin, E4.6; CD44, Hermes 1; P1G12, P3H9; CD43, DF-T1; ICAM-1, P3.58; *c-kit*, 3D6, SR-1. These mAbs were added alone or in combination to the HMC-1 cell suspensions 1 h before the assay (1, 5 and 10 μg/ml at 37° C). HMC-1 cells were seeded on fibroblast monolayers or 96-well plates

Table 1 Monoclonal antibodies for evaluation of cell surface antigen expression and effects in adhesion assays

Specificity	Monoclonal antibody	Species, Ig subclass	Function blocking	Source, reference
β_1-chain	Aj2	Mouse, IgG	–	L. J. Old, Memorial Cancer Center, New York, N. Y. [16]
	4B4	Mouse, IgG1	+	Coulter Electronics, Krefeld, Germany [17]
	mAb13	Rat, IgG	+	S. K. Akayama, Howard Cancer Center, Washington [18]
VLA-1	1B31.1	Mouse, IgA	+	I. Bank, Chaim Sheba Medical Center, Tel Hashomer, Israel
VLA-2	5E8	Mouse, IgG	+	R. B. Bankert, Roswell Park Memorial Institute, Buffalo, N. Y. [19]
	Gi17	Mouse, IgG	–	S. Santoso, Justus Liebig Universität Gießen, Germany
	Gi19	Mouse, IgG	–	S. Santoso
	6F1	Mouse, IgG	+	B. Geller, University of New York, Stony Brook N. Y.
VLA-3	P1B5	Mouse, IgG1	+	Biomol, Hamburg, Germany [20]
	J143	Mouse, IgG	–	L. J. Old [16]
VLA-4	P4G9	Mouse, IgG2b	+	Biomol [21]
VLA-5	P1D6	Mouse, IgG1	+	Biomol [22]
VLA-6	6B4	Mouse, IgG	–	A. Sonnenberg, Netherlands Cancer Institute, Amsterdam, The Netherlands
	MT78	Mouse, IgG	–	C. E. Klein [23]
	GOH3	rat, IgG	+	A. Sonnenberg [24]
β_2-chain	7E4	Mouse, IgG1	+	Dianova, Hamburg, Germany
LFA-1	25.3.1	Mouse, IgG1	+	Dianova
Mac-1	BEAR1	Mouse, IgG1	–	Dianova
p195,95	BU15	Mouse, IgG1	–	Dianova
β_3-chain	AP-3	Mouse, IgG	–	P. Newman, Blood Center of Southeastern Wisconsin, Wis. [25]
α_v-chain	17E6	Mouse, IgG1	+	S. Goodman, Merck, Darmstadt, Germany
	14D9	Mouse, IgG1	+	S. Goodman
	20A9	Mouse, IgG1	–	S. Goodman
	LV230	Mouse, IgG	–	A. N. Houghton, Memorial Cancer Center, New York, N. Y.
VNR	LM609	Mouse, IgG	+	D. Cheresh, Scripps Clinic, La Jolla, Calif [26]
$\alpha_v\beta_5$	P1F6	Mouse, IgG1	+	Biomol
$\alpha_E\beta_7$	28C12	Mouse, IgG	+	M. Brenner [27]
	26F1	Mouse, IgG	+	M. Brenner [27]
	2G9	Mouse, IgG	+	M. Brenner [27]
L-Selectin	Dreg56	Mouse, IgG1	–	Dianova
E-Selectin	1.2B6	Mouse, IgG1	–	Dianova
P-Selectin	CLB-thromb/6	Mouse, IgG1	–	Dianova
ICAM-1	P3.58	Mouse, IgG	+	L. J. Old [16]
ICAM-3	HP2/19	Mouse, IgG2a	+	Dianova
VCAM-1	1G11B1	Mouse, IgG1	–	Cymbus Bioscience, Southampton, UK
NCAM	T199	Mouse, IgG1	–	Dianova
	GC-4	Mouse, IgG1	–	Sigma, St. Louis, Mo.
	VC1.1	Mouse, IgM	–	Sigma
LFA-2	6F10.3	Mouse, IgG1	–	Dianova
LFA-3	AICD58	Mouse, IgG2a	+	Dianova

Table 1 (continued)

Specificity	Monoclonal antibody	Species, Ig subclass	Function blocking	Source, reference
CD44	P1G12	Mouse, IgG1	+	Biomol
	Hermes1	Rat, IgG2a	+	Endogen, Boston, Mass
	P3H9	Mouse, IgG1	+	Biomol
E-Cadherin	HECD-1	Mouse, IgG1	–	Biermann, Bad Nauheim, Gemany
	E4.6	Mouse, IgG	+	K. L. Cepek [28]
P-Cadherin	NCCAD-299	Mouse, IgG	–	A. Ochiai, National Cancer Research Institute, Tokyo, Japan
c-*kit*	95C3	Mouse, IgG1	(+)	Dianova
	3D6	Mouse, IgG2a	+	Boehringer, Mannheim, Germany
	SR-1	Mouse, IgG2a	+	V. Broudy, University of Washington, Seattle, Wash [29]
ε-BP	Anti ε-BP	Goat, IgG	–	F. Liu, Scripps Research Institute, La Jolla, Calif.
	Anti ε-BP	Rabbit, IgG	–	F. Liu
$Fc_{\varepsilon}RI$	15A5	Mouse, IgG	–	R. Chizzonite, Hofmann-La Roche, Nutley, N.J.

coated with the ECM substrates with the blocking mAbs being present during the entire assay. Control assays using isotype-matched mAbs were carried out in parallel.

To determine the effect of Arg-Gly-Asp (RGD) peptides on HMC-1 cell adhesion, excess GRGDTP or GRADSP peptides (1–20 μg each) were added to the HMC-1 cells for 1 h at 37° C before testing adhesion.

To determine whether cell surface carbohydrates are involved in the adhesion of HMC-1 cells to fibroblasts, HMC-1 cells were pretreated with 1–5 μg/ml tunicamycin (Calbiochem, La Jolla, Calif) for 4 h at 37° C. Also, a number of different oligosaccharides were examined for their ability to inhibit HMC-1 cell adhesion. HMC-1 cells were incubated with the following saccharides for 1 h at 37° C: maltose (25 m*M*, 50 m*M*), β-lactose (25 m*M*, 50 m*M*), asialofetuin type I (2.5 mg/ml, 5 mg/ml), N-acetylgalactosamine (25 m*M*, 50 m*M*), N-acetylglucosamine (25 m*M*, 50 m*M*) and methyl-α-D-mannopyranoside (25 m*M*, 50 m*M*) (all from Sigma).

Pretreatment of cells with inhibitors of cell metabolism, signalling pathways and proteinases

The following agents were tested for their effects on adhesion following a 1-h incubation at 37° C at the concentrations indicated: actinomycin D (10, 20, 40, 60, 80 μg/ml), cycloheximide (10, 20, 40, 60, 80 μg/ml) and calphostin C (inhibitor of protein kinases); (0.5, 1, 1.5, 2 μ*M*) (all from Calbiochem, La Jolla, Calif.).

To determine the effect of proteinases on HMC-1 cell attachment to fibroblasts, HMC-1 cells were incubated for 1 h at 37° C with the following proteinases: trypsin (from bovine pancreas), pronase (from *Streptomyces griseus*), endoproteinase Glu-C (from *Staphylococcus aureus* V8) (all from Boehringer, Mannheim, Germany). The cells were then centrifuged, resuspended in RPMI-1640 medium supplemented with 10% FCS to stop proteinase action and seeded on untreated fibroblast monolayers or ECM substrates as described above. In control assays, HMC-1 cells were handled identically except for the presence of proteinase.

Divalent cationic requirements

We sought to determine whether HMC-1 cell adhesion to fibroblasts requires calcium or magnesium ions. EDTA disodium salt (Sigma; 1, 2, 5, 10, 15, 20 m*M*) was added to HMC-1 cell suspensions 15 min before the assay. Because fibroblasts detached from the wells in the presence of EDTA, they were first fixed with paraformaldehyde (0.1% in PBS, pH 7.5) for 20 min at 4° C. The fixative was then washed three times from the cultures with complete medium, and fibroblast cultures were incubated with HMC-1 cells as described above. Control assays were performed both with and without fixation of the fibroblast cultures and in the absence of EDTA.

Cell viability

To exclude cytotoxicity of the experimental procedures, we monitored the viability of fibroblasts, HMC-1 and normal mast cells before and after the tests. Viability was assessed by exclusion of trypan blue and in the case of cultured fibroblasts by examining the integrity of monolayers using an inverted phase contrast microscope. At the concentrations and incubation-times used, none of the treatments (including proteinase treatment) changed the viability as assessed by these methods, so none was considered cytotoxic.

Results

Adhesion of HMC-1 cells to fibroblast monolayers and purified extracellular matrix proteins

Microscopic observation of HMC-1 cells normally growing in suspension revealed that the cells, when seeded on top of a confluent fibroblast monolayer, readily adhere and spread. This has not been seen with leukemia cell lines (HL60, K562) which adhere poorly and show no spreading (data not shown). Since adhesion mechanisms are likely to be of crucial importance for the localization and function of mast cells in peripheral tissues, we investigated in detail, how HMC-1 cells and normal human mast cells isolated from skin adhere to cellular and extracellular components of connective tissue.

In a first series of experiments human and mouse fibroblast monolayers (F135-60-86-skin and BALBc/3T3, respectively) were used as substrates. Adhesion was quantified in a conventional adhesion assay using the fluores-

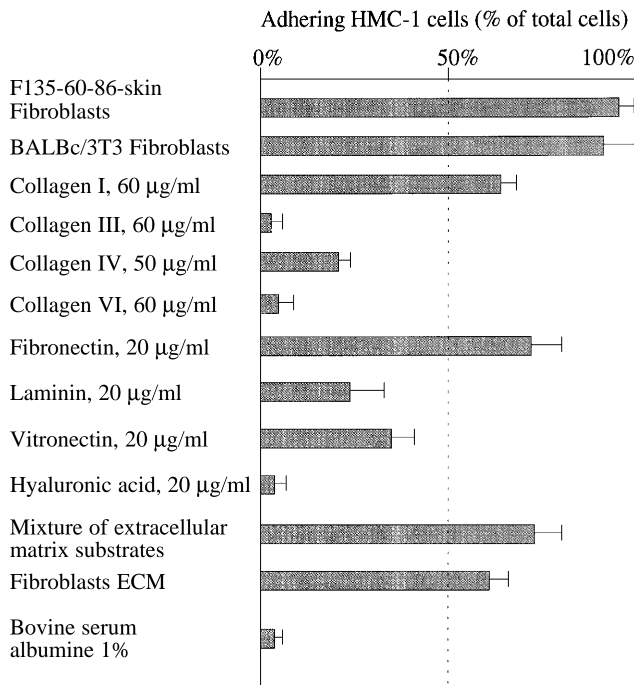

Fig. 1 HMC-1 cell adhesion to fibroblasts and to ECM substrates. The fibroblast monolayers were 90–100% confluent. To determine cell adhesion, 96-well flat bottomed plates were coated with the indicated concentrations of ECM proteins. The adhesion assay was performed as described in Materials and methods. The percentage of adhering cells was calculated as described in Materials and methods. Values are means ± SD of three experiments performed in triplicate. (*Mixture of extracellular matrix substrates* collagen I 60 µg/ml, fibronectin 20 µg/ml, laminin 20 µg/ml, vitronectin 20 µg/ml; *Fibroblasts ECM* ECM proteins laid down by the cultured F135-60-86 fibroblasts as described in Materials and methods)

cent dye PKH2-GL for labelling of the adhering cell population. We regularly found that more than 90% of seeded HMC-1 cells efficiently adhered to the fibroblast monolayer. In a representative experiment, adhesion of HMC-1 cells to F135-60-86-skin and BALBc/3T3 fibroblasts was 96.5 ± 3.1% (mean ± SD) and 90.5 ± 6.3%, respectively (Fig. 1). The adhesion of HMC-1 cells to the endothelial cell line HUVEC-1 was only 22.1 ± 7.0% (data not shown).

To address the question as to whether adhesion of HMC-1 cells to fibroblasts is caused by indirect adhesion mediated by ECM proteins which are secreted and deposited on the fibroblast cell surface or in the extracellular space surrounding them, we sought to determine to which purified ECM proteins HMC-1 cells adhere. HMC-1 cells readily attached to Coll I and FN, to a lesser extent to Coll IV, LM and VN, but not to Coll III or VI or HA. In comparison to their adhesion to fibroblast monolayers, HMC-1 cells adhered less efficiently to ECM components (Coll I 73.9 ± 2.9%, Coll IV 29.4 ± 3.0%, FN 78.9 ± 5.8%, LM 21.9 ± 5.2%, VN 35.3 ± 2.8%, mixture of ECM proteins, i.e. Coll I, FN, VN and LM, 79.2 ± 3.3%). HMC-1 cells did not adhere to Coll III or VI or HA (Fig. 1). The adhesion of HMC-1 cells to the ECM components laid down by the cultured F135-60-86 fibroblasts was 62.5 ± 5.1%.

Anti-integrin mAbs efficiently inhibit mast cell adhesion to ECM components but not to fibroblast monolayers

In order to identify receptors on the surface of HMC-1 cells and on fibroblasts we quantified the surface expression of known adhesion receptors on these cells by flow cytometry using a FACScan. HMC-1 cells expressed several integrin receptors ($\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_v\beta_3$, $\alpha_L\beta_2$ and to a lesser extent $\alpha_5\beta_1$, $\alpha_v\beta_5$) and the cell surface proteoglycan CD44, but no selectins or E- or P-cadherin [32, 33, 34] (Table 2). F135-60-86-skin fibroblasts were also analysed for surface expression of adhesion receptors. They expressed a number of β_1- and α_v-integrins, ICAM-1 and CD44 (Table 3).

Expression of integrin receptors on HMC-1 cells was further confirmed by radioimmunoprecipitation after metabolic labelling. α-chain-specific mAbs immunoprecipitated the α_2-chain and α_3-chain as 160 and 130 kDa species, the α_v-chain as 125 kDa and the α_L-chain as 180 kDa bands (Fig. 2). However, immunoprecipitates employing a mAb directed to the β_1-subunit of integrins,

Table 2 Expression of cell surface molecules on HMC-1 cells, as determined by flow cytometry

Antigen (monoclonal antibody)	Structure	Expression
β_1 (Aj2)	β_1-integrins	+++
α_1 (1B3.1)	VLA-1	–
α_2 (5E8)	VLA-2	+
α_3 (P1B5)	VLA-3	++
α_4 (P4G9)	VLA-4	+
α_5 (P1D6)	VLA-5	+
α_6 (6B4)	VLA-6	+
β_2 (7E4)	β_2-integrins	+
α_L (25.3.1)	LFA-1	++
α_M (BEAR1)	Mac-1	–
α_x (BU15)	p195,95	–
β_3 (AP-3)	β_3-integrins	+
$\alpha_v\beta_3$ (LM609)	VNR	+
$\alpha_v\beta_5$ (P1F6)	β_5-integrin	+
$\alpha_E\beta_7$ (28C12)	β_7-integrin	–
E-Selectin (1.2B6)	Selectine	–
P-Selectin (CLB-thromb/6)		–
L-Selectin (Dreg56)		–
ICAM-1 (P3.58)	Immunoglobulin family	++
ICAM-3 (HP2/19)		++
VCAM-1 (1G11B1)		–
NCAM (T199)		–
LFA-2 (6F10.3)		–
LFA-3 (AICD58)		+++
CD44 (P1G12)		+++
E-Cadherin (HECD-1)	Cadherine	–
P-Cadherin (NCCAD)		–
c-*kit* (SR-1)		+++
15A5 ($Fc_\varepsilon RI$)		–
ε-BP		–

Table 3 Expression of cell surface molecules on F135-60-86-skin cells, as determined by flow cytometry

Antigen (monoclonal antibody)	Structure	Expression
β_1 (Aj2)	β_1-integrins	+++
α_1 (1B3.1)	VLA-1	+
α_2 (5E8)	VLA-2	+
α_3 (P1B5)	VLA-3	+++
α_4 (P4G9)	VLA-4	–
α_5 (P1D6)	VLA-5	++
α_6 (6B4)	VLA-6	–
β_2 (7E4)	β_2-integrins	–
α_L (25.3.1)	LFA-1	–
α_M (BEAR1)	Mac-1	–
α_x (BU15)	p195,95	–
β_3 (AP-3)	β_3-integrins	–
$\alpha_v\beta_3$ (LM609)	VNR	++
$\alpha_v\beta_5$ (P1F6)	β_5-integrin	+
$\alpha_E\beta_7$ (28C12)	β_7-integrin	–
E-Selectin (1.2B6)	Selectine	–
P-Selectin (CLB-thromb/6)		–
L-Selectin (Dreg56)		–
ICAM-1 (P3.58)	Immunoglobulin family	+
ICAM-3 (HP2/19)		–
VCAM-1 (1G11B1)		+
NCAM (T199)		–
LFA-2 (6F10.3)		–
LFA-3 (AICD58)		+
CD44 (P1G12)		+++
E-Cadherin (HECD-1)	Cadherine	–
P-Cadherin (NCCAD)		–

which precipitates all integrin heterodimers bearing the β_1-subunit, revealed a strong band at 100–110 kDa which cannot be explained by the other β_1-chain-associated α-chains which were precipitated by the α-chain specific mAbs. This observation raises the possibility that HMC-1 cells might also express other β_1-chain-associated α-subunits migrating as 100–110 kDa bands under reducing conditions.

We further examined the effect of function-blocking mAbs to integrins and other adhesion receptors (listed above, and see Table 1). The attachment of HMC-1 cells to Coll I, Coll IV, FN, LM and a mixture of ECM proteins was almost completely inhibited by the function-blocking mAb 4B4 directed to the β_1-chain of integrins (Fig. 3). The attachment of HMC-1 cells to the ECM proteins secreted by the F135-60-86 fibroblasts in tissue culture was inhibited by the function-blocking mAb 4B4 from 62.5 ± 5.1% to 17.8 ± 6.5%. Adhesion to VN was blocked by the function-blocking antihuman α_v-integrin mAb 17E6. However, attachment and spreading of HMC-1 cells to fibroblasts was not influenced by function-blocking mAbs directed to integrins, not by those directed to cadherins, CD 44, LFA-3 or ICAM-1. This indicates that HMC-1 cells utilize other adhesion receptors to adhere to fibroblast cell surfaces.

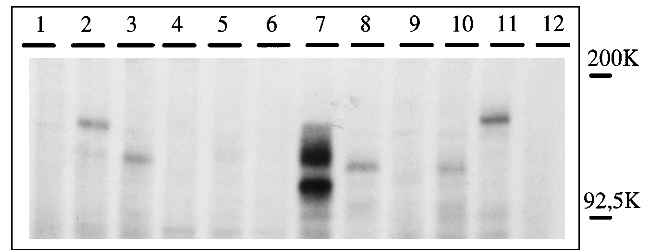

Fig. 2 Synthesis of adhesion receptors in HMC-1 cells. Con A-bound fractions of NP-40 cell lysates from ^{35}S-methionine-labelled cultures of HMC-1 cells were analysed by immunoprecipitation, SDS-PAGE on 9% polyacrylamide gels and autoradiography. The figure shows immunoprecipitates obtained with mAbs recognizing different integrin chains: *lane 1* VLA-1 (1B3.1); *lane 2* VLA-2 (5E8); *lane 3* VLA-3 (J143); *lane 4* VLA-4 (P4G9); *lane 5* VLA-5 (P1D6); *lane 6* VLA-6 (MT78); *lane 7* β_1-chain (Aj2); *lane 8* α_v-chain (LV230); *lane 9* $\alpha_v\beta_3$ VNR (LM609), *lane 10* β_5-integrin (P1F6); *lane 11* LFA-1 (25.3.1); *lane 12* mouse control. Note, the strong band at 110 kDa precipitated by the β1-chain mAb Aj2

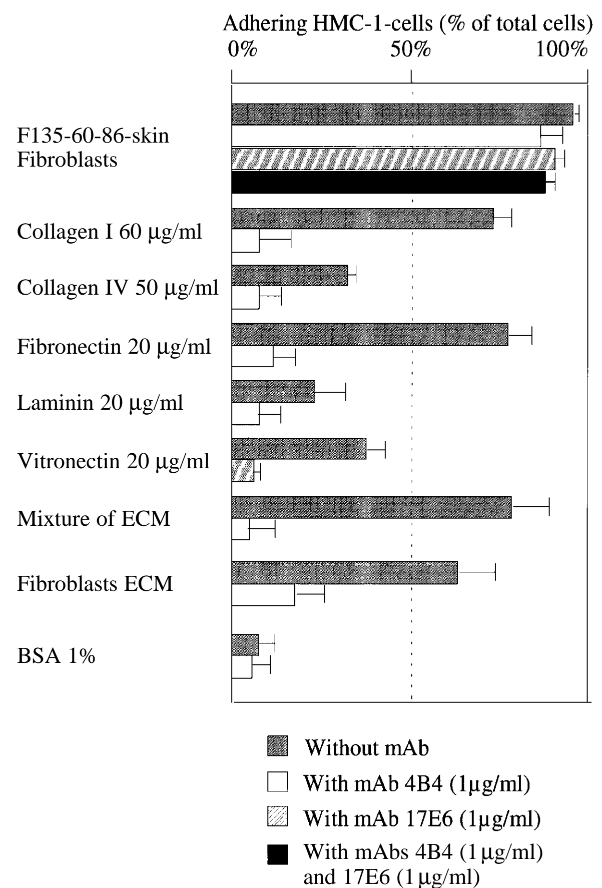

Fig. 3 Inhibition of HMC-1 cell adherence to ECM substrates by the function-blocking anti-integrin antibodies 4B4 and 17E6. Fibroblasts were cultured to 90–100% confluence, the wells were coated with ECM substrates at the concentrations indicated. Values are means ± SD of three experiments performed in triplicate (*Mixture of ECM* collagen I 60 µg/ml, fibronectin 20 µg/ml, laminin 20 µg/ml, vitronectin 20 µg/ml; *Fibroblasts ECM* ECM proteins laid down by the cultured F135-60-86 fibroblasts as described in Materials and methods)

To determine whether the adhesion of HMC-1 cells to fibroblasts is mediated through an RGD-dependent receptor, we added the GRGDTP peptide to the adhesion assay. HMC-1 cell adherence to fibroblasts was not inhibited (data not shown).

Previous studies employing mouse cells have shown that mast cell growth factor (stem cell factor, SCF) exposed and bound on the fibroblast cell surface by itself can alone mediate mast cell adhesion to fibroblasts by engaging their c-*kit* receptor [35]. Our inhibition experiments introducing excess amounts of soluble SCF of the blocking anti-c-*kit* mAbs 3D6 and SR-1 showed that HMC-1 cells and human mast cells do not utilize this pathway (data not shown). To determine whether certain cell surface carbohydrates are responsible for adhesion of HMC-1 cells to fibroblasts, we pretreated the cells with tunicamycin and introduced several saccharides known to block lectin interactions into the adhesion assay medium. Pretreatment with tunicamycin and all saccharides tested did not reduce the adhesion of HMC-1 cells to fibroblasts (data not shown).

Adhesion of HMC-1 cells to fibroblasts is partially dependent on calcium and the PKC pathway, requires protein synthesis and is partially sensitive to proteinases

Treatment with inhibitors of protein or RNA synthesis, cycloheximide and actinomycin D, decreased the attachment of HMC-1 cells in a dose-dependent manner to 53.3 ± 2.5% and 59.1 ± 5.7% (Fig. 4). Treatment with calphostin C, a specific inhibitor of PKC-α, -β and -γ, decreased the adhesion of HMC-1 cells to fibroblasts to 54.9 ± 5.8%. The effects observed indicate that protein and RNA de novo synthesis as well as PKC activity are at least partially required for the adhesion of mast cells to fibroblasts.

Pretreatment of HMC-1 cells with trypsin (up to 10 μg/ml for 1 h) and pronase (up to 250 μg/ml for 1 h) decreased HMC-1 cell adhesion to fibroblasts to 51.3 ± 5.4% and 45.6 ± 7.7%, respectively (Fig. 4). Pretreatment of HMC-1 cells with the endoproteinase Glu-C (V8, up to 250 μg/ml) had no effect. Preincubation with EDTA did not augment trypsin sensitivity (data not shown).

Adhesion of HMC-1 cells to cultured fibroblasts partially required the presence of divalent cations. Only 65.7 ± 4.9% of HMC-1 cells attached in the presence of 2 m*M* EDTA versus 95.2 ± 3.8% cultured without EDTA (Fig. 4). Fixation of the fibroblast cultures with 0.1% paraformaldehyde, which was required for this adhesion assay, did not alter HMC-1 adhesion (96.3 ± 4.2% on fixed fibroblast monolayers versus 95.2 ± 3.8% on nonfixed cultures).

Adhesion of normal human skin mast cells to cultured fibroblasts and Coll I

To determine whether normal mast cells prepared from human foreskin undergo similar adhesive interactions to HMC-1 cells, mast cells isolated from human foreskin were subjected to adhesion tests on fibroblasts as well as Coll I. The fraction of attached normal mast cells was assessed by measuring the total amount of histamine released from the adherent cell population after cell lysis. Pilot experiments revealed that the majority of these mast cells (64.3 ± 3.4%) adhered to fibroblasts, whereas only 38.3 ± 2.9% adhered to Coll I. The effects of the mAbs 4B4 and 17E6, which had inhibited HMC-1 cell adhesion towards Coll I and VN, were also studied. Interestingly, attachment of normal mast cells to Coll I was also blocked by the mAb 4B4 (from 38.3 ± 2.9% to 19.1 ± 4.0%), (Fig. 5). However, no inhibition of mast cell adherence to fibroblasts was noted by blocking β_1- and α_v-chain antibodies, indicating that normal human skin mast cells, such as HMC-1 cells, utilize different adhesion mechanisms for adhesion to fibroblast surfaces than to the ECM components studied.

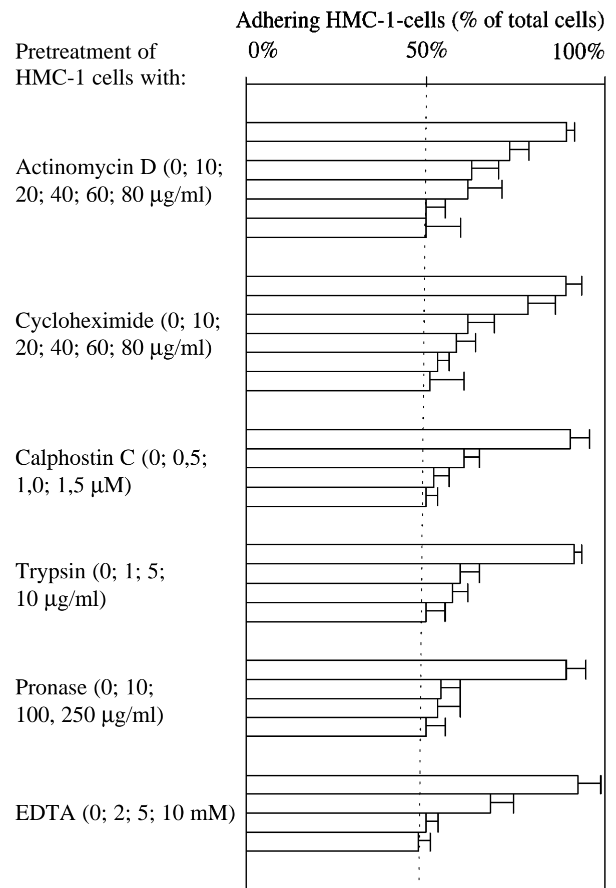

Fig. 4 Partially inhibitory effect of pretreatment with actinomycin D, cycloheximide, calphostin C, divalent cations and proteinase on adhesion of HMC-1 cells to cultured fibroblasts. The HMC-1 cells were treated before the adhesion assay with these agents as described in Materials and methods. The concentrations are indicated. Before the adhesion assay EDTA was added to the HMC-1 cell suspension. Because fibroblasts detached from the wells in the presence of EDTA they were first fixed with paraformaldehyde as described in Materials and methods. Values are means ± SD of two experiments performed in triplicate

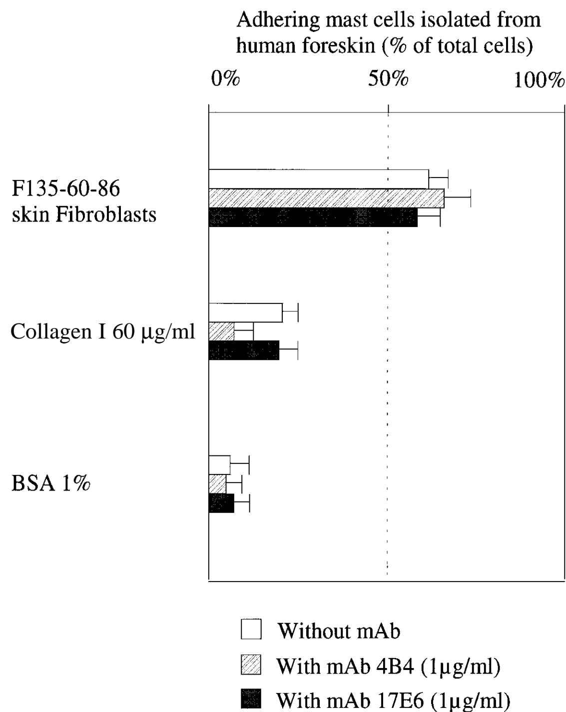

Fig. 5 Adhesion of mast cells isolated from human foreskin to fibroblasts and Coll I. The fibroblast monolayers were 90–100% confluent. To determine cell adhesion, 96-well flat bottomed plates were coated with the indicated concentration of Coll I. The adhesion assay was performed as described in Materials and methods. The percentage of adhering cells was calculated as described in Materials and methods. Values are means ± SD of one experiment performed in triplicate

Discussion

In this study we demonstrated that the human mast cell leukemia cell line HMC-1 and normal human mast cells isolated from foreskin adhere and spread on top of fibroblast monolayers with high efficiency. We also showed that adhesion of mast cells to fibroblasts is not mediated by attachment of mast cells to common ECM proteins like FN and Coll I secreted and deposited by fibroblasts on their extracellular plasma membrane or in intercellular spaces. This became evident because mAbs blocking β_1- or α_v-integrin function did not inhibit mast cell attachment to fibroblasts, although they were very efficient in preventing their attachment to the corresponding ECM components. The results indicate that heterotypic adhesion of human mast cells to fibroblasts must be mediated by different receptors than adhesion to common ECM substrates.

A variety of ECM substrates, including Colls I, III, IV and VI, FN, VN, LM and HA were examined for their ability to mediate HMC-1 cell attachment. The effects of Coll I on the attachment of normal mast cells isolated from human tissue were also examined. It was found that HMC-1 cells readily adhere to Coll I and FN, to a lesser extent to Coll IV, LM and VN, but not to Coll III or VI or HA. Mast cells isolated from human tissue also adhered to Coll I. In this regard our results are not in accordance with data from the mouse species. Thompson et al. found that Colls I and IV and FN were ineffective in promoting mast cell attachment, whereas 30–40% adherence of the mouse mast cell line PT18 cells or PMA-stimulated bone marrow-derived mast cells to LM was observed [36]. In accordance with our results, these and other authors have noted changes in cellular morphology (spreading) after attachment of mast cells to ECM components [35, 36]. Bianchine et al. have demonstrated that IL-3-dependent bone marrow-derived mouse mast cells readily adhere to plate-bound VN (60–70%) without previous stimulation [37]. The comparison of attachment to VN, FN and LM revealed that adhesion to VN was greater than to FN (30–40%) or LM (40–50%). These data and also our results indicate that normal matured mast cells may attach to substrates without additional stimuli via PKC- or Fc_{ε}RI-mediated pathways. Kinashi and Springer have reported that the Il-3-dependent mouse mast cell line MC/9 adheres to FN (40%), LM (25%) and VN (15%) [38]. In that study, bone marrow-derived mouse mast cells stimulated with steel factor (SCF) or PMA showed no significant increase in adhesiveness to these substrates. However, Dastych and Metcalfe demonstrated that SCF may augment adhesion to FN [39]. Versano et al. studied the attachment of dog mastocytoma cells to tracheal epithelial cells and various ECM substrates [40]. Although readily adhering to epithelial cells (approx. 35%) the mastocytoma cells attached poorly to Colls I and IV and FN.

In contrast to the studies mentioned above we analysed human mast cells not only for their attachment characteristics but also for their surface expression of a large number of adhesion receptors. In accordance with other studies, we found that HMC-1 cells expressed cell surface molecules in a similar manner to tissue mast cells [32–35]. The mast cell line HMC-1 expresses a number of β_1- and α_v-integrins. These integrins permit the binding of cells to ECM molecules such as LM, VN, Coll and FN. Since we showed that function-blocking mAbs to β_1- and α_v-chains of integrins prevented mast cell attachment to these ECM compounds, the receptors are clearly in a functionally active state. In contrast, although HMC-1 cells highly express the standard form of the CD44 HA receptor, they did not attach to HA, suggesting that this receptor is not activated. Analysis of cell surface expression as well as synthesis studies have indicated that HMC-1 cells express the following β_1-integrins: $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$. Interestingly, in immunoprecipitation experiments employing mAbs directed to the β_1-chain a prominent band with a molecular weight of approximately 105 kDa was coprecipitated. The identity of this band is currently not known. Its molecular weight is compatible with that reported for the α_7-chain but not for the α_8- and α_9-chains. Alternatively, human mast cells may express a β_1-chain variant. Under the experimental conditions used in these experiments, it is unlikely that the 105 kDa species represented an artefactual breakdown product. However, since mAbs, which block the function of all known β_1-integrins, did not reduce the adhesion of mast cells to fibroblasts, it

is not possible that a β_1-integrin containing a 105 kDa α-chain species was responsible for the observed heterotypic cell-cell interaction. Integrin $\alpha_E\beta_7$, recently found to mediate heterotypic cell-cell interaction [28] was not observed on HMC-1 cells.

SCF-c-*kit* interactions are known to be crucial for differentiation of human mast cells. Recently, Adachi et al. postulated that the c-*kit* receptor may also operate as an adhesion molecule by permitting attachment to SCF expressed on fibroblast cell surfaces in its transmembrane form [35]. From studies of mouse mast cells, Adachi et al. suggested that SCF-c-*kit* interactions are necessary for attachment, proliferation and survival of mast cells. Our experiments employing excess amounts of soluble SCF as well as mAbs blocking the SCF binding site of c-*kit* indicated that attachment of human mast cells to fibroblasts is not mediated by SCF c-*kit* interaction.

Cadherins are known to mediate heterotypic cell-cell interactions [41, 42]. Most cadherins are resistant to trypsin activity under physiological conditions, and become sensitive after depletion of cations (e.g. by pretreatment with EDTA). We found that the sensitivity of mast cell-fibroblast adhesion to trypsin pretreatment (≈ 50%) was not increased by EDTA, suggesting that cadherins are not involved. Furthermore, we did not find expression of E- and P-cadherins on HMC-1 cells.

Oligosaccharides expressed on cell surface glycoproteins may act as ligands for specific cell-cell adhesion receptors, e.g. selectins [43]. However, E-, P- and L-selectins, which are essential during the initial stages of leucocyte recruitment and extravasation, were not expressed on HMC-1 cells or fibroblasts. Furthermore, pretreatment with tunicamycin or administration of excess amounts of several other oligosaccharides different from sialyl Le^x and sialyl Le^a (see Materials and methods) had no inhibitory effect on mast cell-fibroblast attachment. Based on these results, we postulate that yet unidentified receptors mediate adhesion of mast cells to fibroblasts.

Mast cell adhesion to fibroblasts partially requires divalent cations as well as RNA and protein synthesis. The fact that heterotypic adhesion can be reduced to approximately 50% but not further by the presence of EDTA or by preincubation with trypsin may suggest that two different receptor-ligand pairs are operating. Furthermore, pretreatment of HMC-1 cells with actinomycin D, cycloheximide and calphostin C only partially reduced mast cell attachment to fibroblasts even with maximal dosages.

Hopefully, further studies will permit the molecular characterization of the receptors involved and may provide new insights into the function of normal differentiated mast cells in tissues.

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