Mast Cells Induce T-Cell Adhesion to Human Fibroblasts by Regulating Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 Expression

Hong Meng,* Mary J. Marchese,* Jonathan A. Garlick,† Ante Jelaska,‡ Joseph H. Korn,‡ James Gailit,* Richard A. F. Clark,* and Barry L. Gruber*

Departments of Medicine,* Dermatology, and †Oral Biology and Pathology, School of Medicine and ‡School of Dental Medicine, SUNY at Stony Brook, Stony Brook, New York, and Department of Veterans Affairs Medical Center, Northport, New York; and ‡Arthritis Center, Department of Medicine, Boston University Medical Center and Boston Veterans Administration, Boston, Massachusetts, U.S.A.

The capacity of mast cell products to mediate T-cell adhesion to fibroblasts was explored using heterotypic coculture systems or by exposing fibroblasts to mast-cell-conditioned media (MCCM), prepared by degranulating mast cells with calcium ionophore. Experimental results indicated that fibroblasts exposed to MCCM for 24 h bound fivefold more T cells than control fibroblasts. Binding was inhibited with intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) neutralizing antibodies. Enzyme-linked immunosorbent assay and fluorescence-activated cell sorter analysis revealed that fibroblasts exposed to MCCM markedly increased ICAM-1 and VCAM-1 surface expression by 4 h, with levels maximal at 16 h and returning toward baseline by 48 h. A dose-dependent response of ICAM-1 and VCAM-1 expression was noted using serial dilutions of MCCM or by altering the ratio of degranulated mast cells cocultured with fibroblasts.

Recently, a plethora of studies have emerged that depict the molecular mechanisms underlying the emigration of circulating cells into peripheral tissue sites during inflammation [1–3]. Most investigators have focused on events early in the process of leukocyte recruitment, thus not addressing the question of how leukocyte accumulation is sustained in the extravascular space at a site of inflammation. Endothelial cells lining the microvasculature are critical to early events by expressing adhesive molecules in response to inflammatory signals. We have previously demonstrated [4] that mast cells activate microvascular endothelial cells, leading to enhanced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These events may be operative when mast cells contact an allergen and initiate an inflammatory reaction. We now extend these observations to probe how mast cells might sustain an inflammatory reaction, as in a late-phase allergic response. A mechanism by which this might occur is up-regulation of the adhesive properties of fibroblasts in extravascular sites. Therefore, we studied the capacity of mast cell products to induce both lymphocyte adherence to fibroblasts and ICAM-1 and VCAM-1 expression. Our data show that mast cells mediate lymphocyte attachment to fibroblasts by up-regulating ICAM-1 and VCAM-1 on the surface of fibroblasts.

MATERIALS AND METHODS

Materials Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and Fungizone were purchased from Gibco Laboratories (Grand Island, NY). Percoll, calcium ionophore A23187, histamine, and heparin were purchased from Sigma (St. Louis, MO). Mouse monoclonal antibody (MoAb) specific for VCAM-1 was obtained from Genzyme (Cambridge, MA). R1/1 MoAb, which recognizes ICAM-1, was generously provided by Dr. Robert Rothlein at

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Boehringer Mannheim (Ridgefield, CT). No cross-reactivity exists in the horse anti-mouse IgG and streptavidin-conjugated horse radish peroxidase (Durham, NC); control mouse ascites fluid was from Sigma; biotinylated horse anti-mouse IgG and streptavidin-conjugated horseradish peroxidase were from Vector (Burlingame, CA). Polyclonal rabbit anti-mouse tumor necrosis factor-α (TNF-α) neutralizing antibody, murine TNF-α, interleukin (IL)-1β, IL-4, and human T-cell enrichment column were purchased from R&D Systems (Minneapolis, MN). Hamster monoclonal B122 anti-murine IL-1β was kindly provided by Dr. David Chaplin at Howard Hughes Medical Institute (St. Louis, MO).

The ability of antibody B122 to neutralize the biologic activity of murine rIL-1β has been verified using the D10 costimulation assay [5]. The ability of neutralizing antibody to inhibit rTNF-α-induced ICAM-1 and VCAM-1 expression on fibroblasts was tested. The antibody was capable of inhibiting 93% to 95% of rTNF-α-induced ICAM-1 and VCAM-1 expression on human dermal fibroblasts. The antibody was also capable of inhibiting the expression on fibroblasts of murine TNF-α, as determined by standard cytotoxicity bioassay using L929 cells [6].

Cell Culture Normal human dermal fibroblasts isolated from adult dermis were obtained from the National Institute on Aging, Aging Cell Culture Repository, Coriell Institute for Medical Research (Camden, NJ), designated AG05838A and AG04145. All fibroblasts were cultured in DMEM with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml Fungizone ( Gibco). DMEM with 0.1% high-purity bovine serum albumin (medium A) was used in the experiments described below.

In some experiments, normal human adult lung fibroblasts and synovial fibroblasts were also used for comparing the results of fibroblasts from different organ tissues. Human lung fibroblasts, designated AG02603, were obtained from the same source as above. These cells were also cultured in DMEM with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml Fungizone (Gibco). Human synovial fibroblasts were isolated from normal synovium of adult patients undergoing joint surgery as a result of accidental trauma. Human synovial fibroblasts were cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml Fungizone (Gibco).

Preparation of Mast-Cell-Conditioned Media (MCCM) Bone-marrow–derived IL-3–independent murine mast cells (CI–MC/C57; 1; kindly supplied by Dr. S. Galli, Boston, MA) [6] were maintained in DMEM with 10% FBS, 2 mM L-glutamine (Gibco), and 500 mM 2-hydroxyethylmercaptan (Sigma). To prepare mast cell releasates, mast cells in fresh culture medium were depleted by exposure to the mast cell secretagogue calcium ionophore A23187. Optimal stimulation and release were acquired by incubating mast cells (5 x 10^6/ml) with 500 ng/ml A23187 for 6 h at 37°C. Mast cell supernatants were aliquoted and stored at −70°C to be used for future experiments. Rat peritoneal mast cells were also used for preparing mast cell releasates of a connective tissue phenotype. Rat peritoneal mast cells were freshly isolated by a Percoll density gradient technique essentially as described by Enerbäck and Svensson [7]. Histamine secretion was quantitated by a double radioimmunoassay [8] to monitor mast cell degranulation.

Preparation of Human Lymphocytes Peripheral blood mononuclear cells were obtained from normal donors of both sexes and prepared by centrifugation on density gradients of Ficoll-Hypaque (Pharmacia). Cells were washed in supplemented Eagle’s minimal essential medium (Gibco) and then passed over a T-cell enrichment column (R&D Systems). The resulting cell population consisted of highly enriched T cells of 93% to 95% purity, with contaminating cells consisting of natural killer cells, as confirmed by surface marker staining and fluorescence-activated cell sorter (FACS) analysis. These preparations were used as the source of cells for adherence studies, as described below.

Adhesion Protein Enzyme-Linked Immunosorbent Assay (ELISA) Confluent cultures of fibroblasts in 96-well plates (Falcon) were incubated at 37°C for specified periods of time with varying concentrations of either MCCM or intact mast cells exposed to the degranulating agent, A23187. Controls consisted of treatment with calcium ionophore alone or culture medium alone. Cell monolayers were washed twice with serum-free culture medium containing 0.1% bovine serum albumin (medium A) and incubated with MoAbs (2 µg/ml) to ICAM-1 or VCAM-1 diluted in medium A for 60 min at 37°C. The cells were then washed three times and incubated for 30 min at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG antibody diluted in medium A. The wells were again washed, and the binding of antibodies was assessed by the addition to each well of 100 µl of tetramethylbenzidine peroxide substrate (Kirkgaard & Perry, Gaithersburg, MD) and deionized water (Sigma). The reaction was stopped by the addition of 100 µl of 0.08 N H₂SO₄; absorbance was measured at 450 nm on an ELISA reader (Thermofax; Molecular Devices, Menlo Park, CA) interfaced with Macintosh software. Controls consisted of an irrelevant isotype-matched monoclonal antibody (MOPC, IgGl; Sigma) and deletion of the primary MoAbs. All ELISA data expressed represent the mean of several experiments, each performed in triplicate. Statistically significant increases in expression over baseline were analyzed by analysis of variance.

FACS Analysis of Adhesion Molecules Fibroblasts were grown to confluence in T-75 flasks (Falcon) and then stimulated with MCCM for specific time periods. Controls consisted of treatment with calcium ionophore alone or culture medium alone. Cells were harvested with cell dissociation buffer (Gibco), washed twice in medium A, and incubated for 10 min at 37°C for 45 min on ice. Cells were then incubated with primary MoAbs for 60 min at 4°C. Irrelevant pooled mouse IgG (Sigma) was used as a control. After three washes with cold medium A, the conjugated sheep anti-mouse IgG (6 µg/ml) was added for another 60-min incubation at 4°C. The cells were washed exhaustively with cold phosphate-buffered saline (PBS) and resuspended in PBS to yield 2 x 10⁶ cells/ml. The cell surface expression was quantitated by flow cytometry performed with a FACStar Plus cell sorter (Becton Dickinson Immunochemistry Systems, San Jose, CA), and the data were analyzed using LYSYS II software on an HP 340 computer workstation (Hewlett Packard, Fort Collins, CO).

T-cell Adhesion Assay The assay was performed essentially as described by Piela-Smith et al. [9]. Briefly, confluent fibroblasts in 96-well plates were stimulated with MCCM, as described above. The cells were then washed with complete medium prewarmed to 37°C and 100 µl of purified T-lymphocyte suspension (4 x 10⁶/ml) was added. After a coinubation period of 1 h at 37°C to allow the T lymphocytes to bind to fibroblasts, nonadherent T cells were removed by gentle washing of the monolayers with medium A, prewarmed to 37°C. In some instances, neutralizing MoAbs to ICAM-1 or VCAM-1 were added to fibroblast cultures for 60 min at 37°C before the addition of T cells.

Adherent T cells were quantitated by microscopic examination after cell monolayers were air dried and stained with 1% methylene blue. Four random fields in each well were counted using an ocular grid. Adhesion was expressed as the number of adherent T cells per grid. Results are expressed as the mean of triplicate wells.

Northern Hybridization The VCAM-1 cDNA (kindly provided by Dr. C.M. Ballantyne, Houston, TX) was isolated as approximately a 1000-bp base-pair PstI fragment and directly labeled in 1% low-melt agarose by random hexamer priming using [3P]dCTP (DuPont NEN, Boston, MA) [10]. The ICAM-1 mRNA probe was pG4H1.1ms-plasmid (kindly provided by Dr. H. Springer, Center for Blood Research, Boston, MA). The KpnI-SafI 1.5-kb fragment insert was labeled by the random primer kit (DuPont NEN). Specific activity ranged from 1 x 10⁶ to 1 x 10⁷ cpm/µg DNA. Confluent fibroblasts were treated with MCCM or media alone for the period of time indicated. Total cellular RNA was extracted using guanidine isothiocyanate and phenol-chlororoform-isooamyl alcohol [11]. Equal amounts of total RNA (20 µg) from each sample were subjected to electrophoresis on a 1.2% agarose-formaldehyde gel and transferred to nitrocellulose [12]. The membrane was prehybridized for 4 h at 42°C (50% formamide, 5 x Denhardt’s, 0.2% sodium dodecysulfate, 50 µg/ml bovine salmon sperm DNA, 5 x standard saline citrate, 50 mM HEPES, 5 mM ethylenediaminetetraacetic acid, and 10 µg/ml poly-A). Overnight hybridization was performed at 42°C in the same solution containing radiolabeled probe. The membrane was washed to a final stringency condition of 0.1 x standard saline citrate/0.1% sodium dodecylsulfate at 68°C for 1 h and then exposed to Kodak X-OMAT film at −70°C with an intensifying screen for 24 to 48 h. When appropriate, the nitrocellulose filters were stripped by boiling, and the adequacy of stripping was confirmed by overnight autoradiography at −70°C before rehybridization with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Densitometry was performed using the IMAGE software system.

Organotypic Skin-Equivalent Culture Model Human foreskin keratinocytes were cultured on human dermal foreskin fibroblasts in organotypic coculture to generate skin equivalents, as described previously [13]. Mast cells were mixed with fibroblasts in a ratio of 1:4 in collagen type I matrix (Organogenesis, Canton, MA) to form a composite culture in collagen. Keratinocytes, seeded on this dermal equivalent, stratified and formed a fully differentiated epithelial layer. Controls consisted of fibroblasts in collagen in the absence of mast cells, treated otherwise in an
identical manner including exposure to mast cell secretagogue A23187. The organotypic model was cultured for 7 d at the air interface to allow complete stratification and differentiation of surface keratinocytes, after which calcium ionophore A23187 was added to the media. After overnight incubation, the organotypic cultures were snap frozen in liquid nitrogen in embedding compound (TBS, Durham, NC) and stored at −70°C.

**Immunoperoxidase Studies of ICAM-1 Expression in the Skin-Equivalent Model** Specimens were processed by preparing 7-μm cryostat sections, which were then air dried and fixed in acetone before rehydration in PBS. After 60 min preincubation with 5% horse serum, tissue sections were reacted with primary MoAbs directed against ICAM-1 (kindly supplied by Robert Rothlein, Boehringer Ingelheim, Ridgefield, CT) or VCAM-1 (R&D) at room temperature for 60 min. After three washes with PBS plus 0.1% bovine serum albumin, biotinylated horse anti-mouse IgG (15 μg/ml) was added for 30 min. The slides were again washed before incubation with streptavidin conjugated to alkaline phosphatase (Vector) for 30 min. The specimens were visualized after the addition of fast red substrate (Biogenix, San Ramon, CA). The sections were then counterstained with hematoxylin before dehydration and mounting. Control experiments deleting the primary MoAbs were performed routinely to allow assessment of endogenous avidin-binding activity and nonspecific reactivity.

**RESULTS**

**MCCM Induces Lymphocyte Attachment to Human Dermal Fibroblasts** A cell adhesion assay was performed to determine the functional consequences of exposure of human dermal fibroblasts to MCCM. To investigate the effect of mast cell products on fibroblast cell adhesion, we added MCCM (previously prepared by degranulating mast cells with calcium ionophore) to confluent fibroblasts in culture. After defined time intervals, the fibroblasts were washed with fresh culture medium, and purified human T cells were incubated with human dermal fibroblasts. After an additional hour, the nonadherent T cells were removed by washing. As shown in Fig 1, exposure of human dermal fibroblasts to MCCM resulted in increased lymphocyte adherence, with maximal values observed by 24 h. MCCM induced approximately fivefold increases in T-cell adherence to human dermal fibroblasts.

To assess the contribution of ICAM-1 and VCAM-1 to T-cell adhesion after MCCM stimulation of fibroblasts, we used MoAbs to ICAM-1 and VCAM-1 for blocking experiments. MCCM-treated human dermal fibroblasts were preincubated with MoAbs to either ICAM-1 or VCAM-1 at 37°C for 60 min before addition of T cells. T-cell adhesion to human dermal fibroblasts could be markedly diminished by antibody to either ICAM-1 or VCAM-1, and a combination of these antibodies yielded further inhibition (Fig 2). Control isotype-matched monoclonal antibodies had no effect on the T-cell binding. MCCM prepared by culturing the mast cells in the absence of any mast cell secretagogue had minimal effect on stimulating T-cell binding (Fig 2). Essentially all the cultured fibroblasts bound numerous T cells to the surface after exposure to MCCM, as shown in Fig 3.

**Cocultured Mast Cells and Fibroblasts or MCCM Increased Fibroblast Expression of ICAM-1 and VCAM-1** Confluent human dermal fibroblasts on 96-well microtiter plates were cocultured with mast cells, which subsequently were degranulated by the addition of calcium ionophore (500 ng/ml). The induction of ICAM-1 and VCAM-1 was determined at 16 h by ELISA. Elevated cell surface expression of ICAM-1 and VCAM-1 adhesion proteins was observed on human dermal fibroblasts cocultured with degranulated mast cells (Fig 4). Calcium ionophore alone had no direct effect on human dermal fibroblasts. A dose-dependent response was noted by varying the ratio of mast cells to fibroblasts in coculture. At a ratio as low as 1:1000 mast cells to fibroblasts, ICAM-1 and VCAM-1 induction was noted, although statistically significant increased expression (p < 0.05) was reached at ratios of 1:100 and higher (Fig 4). Mast cells cocultured with human dermal fibroblasts in the absence of the calcium ionophore failed to induce adhesion molecule expression. Furthermore, mast cells themselves (in the absence of fibroblasts) demonstrated no surface staining for human ICAM-1 or VCAM-1, using these MoAbs (data not shown).

A time-course experiment of adhesion molecule expression after exposure to MCCM was performed, using ELISA to monitor the cell surface expression. Maximal expression was noted between 16 and 24 h, with approximately 120% and 460% increases (p < 0.05) of ICAM-1 and VCAM-1, respectively, over baseline levels on human dermal fibroblasts (Fig 5A). The expression of VCAM-1 in particular then declined at 48 h but was still maintained above the control level (p < 0.05) (Fig 5). Similar kinetic curves were observed using human synovial fibroblasts, with approximately 85% and 450% maximal increases (p < 0.05) of ICAM-1 and VCAM-1, respectively (Fig 5B). The induction of ICAM-1 (41%) and VCAM-1 (85%) on human lung fibroblasts by MCCM was less prominent (p > 0.05) as compared with dermal or synovial fibroblasts (Fig 5C). The mast cell secretagogue A23187 had no
Figure 3. T-cell adhesion to cultured fibroblasts treated with MCCM. T cells were allowed to attach to unstimulated human dermal fibroblasts (A) or fibroblasts after exposure to MCCM (1:10) for 24 h (B) and then washed. Only adherent T cells are observed (arrow). The adhesion of T cells to confluent human dermal fibroblasts was inhibited by the addition of antibody to either ICAM-1 (C) or VCAM-1 (D). Bar, 360 μm.

direct effect on the expression of these adhesion molecules (data not shown). The kinetics and dose dependency of MCCM-induced enhancement of T-cell adhesion were therefore similar to the cell surface expression of adhesion proteins in human dermal fibroblasts, with significant increases seen as early as 4 h (p < 0.05) and declining responses by 48 h (Figs 1, 5). Control isotype-matched MoAbs revealed background binding similar to that of unstimulated fibroblasts (data not shown).

The induction of ICAM-1 and VCAM-1 occurred in a concentration-dependent manner, as determined by the addition of stepwise dilutions of MCCM to fibroblasts (Figs 4, 5). A trend of increase was detected when MCCM was added to human dermal fibroblasts at a concentration as low as 1:100, although a significant increase was observed at 1:20 (p < 0.05). This dose dependency was not as evident on human lung fibroblasts as on dermal and synovial fibroblasts. Under these experimental conditions, constitutive expression of ICAM-1 was higher than that of VCAM-1, especially on human dermal fibroblasts and human synovial fibroblasts.

FACS Analysis of Mast-Cell-Induced ICAM-1 and VCAM-1 Expression on Fibroblasts The effect of MCCM on the cell surface expression of ICAM-1 and VCAM-1 on human dermal fibroblasts was also studied by FACS assay (Fig 6). Confirming the ELISA studies, human dermal fibroblasts constitutively expressed ICAM-1 (Fig 6B). At 24 h, incubation with MCCM resulted in a 10-fold increase of ICAM-1 expression and a sixfold increase of VCAM-1 expression on human dermal fibroblasts (Fig 6C).

Figure 4. Coculture of human dermal fibroblasts with degranulated mast cells induced ICAM-1 and VCAM-1 expression in human dermal fibroblasts. The number of mast cells (MC) added to confluent human dermal fibroblasts (HDFb) was varied to provide effector:target cell ratios as depicted. Mast cells were then degranulated using Calcium ionophore (500 ng/ml). ICAM-1 and VCAM-1 expression at 16 h was measured by ELISA. Data represent the mean ± SEM of two experiments performed in triplicate.
Determination of the Active Component of MCCM  In an attempt to determine which of the potential factors secreted by mast cells was responsible for the effects observed on fibroblasts, we added several well-characterized mast cell products directly to cultured human dermal fibroblasts. ICAM-1 and VCAM-1 were then measured at 24 h by ELISA. The results indicated that histamine (Fig 7) and heparin (data not shown) had no significant effect on adhesion molecule expression. Recombinant IL-1β and TNF-α gave the expected results of ICAM-1 up-regulation (p < 0.05); they caused only slight, and statistically nonsignificant, up-regulation of VCAM-1 compared with baseline (Fig 7). IL-4 had no significant effect on ICAM-1; however, it induced prominent VCAM-1 expression (p < 0.05) on human dermal fibroblasts.

To determine the responsible cytokine within the crude MCCM that stimulated the adhesion molecule expression, we pretreated MCCM with neutralizing antibodies to TNF-α, IL-1β, or IL-4 and measured the effect on human dermal fibroblasts. TNF-α neutralizing antibody was capable of inhibiting 85% of the ICAM-1 but only 53% of the VCAM-1 response (Fig 8). Incubation of MCCM with antibodies to TNF-α and IL-1β together or the combination of antibodies to TNF-α and IL-4 was no more effective than TNF-α neutralizing antibody alone. Neutralizing antibody to IL-1β alone gave 40% and 20% inhibition, respectively, of ICAM-1 and VCAM-1 induction. Neutralizing antibody to IL-4 alone inhibited 20% of ICAM-1 and showed no inhibition of VCAM-1 induction. Hence, it appears that TNF-α is largely responsible for increased ICAM-1 expression, but this mast-cell-derived cytokine is only partially responsible for VCAM-1 induction. In either case, studies using human mast-cell–derived cytokines will be required to gather more information concerning endothelial cell activators relevant to inflammation of the human microvasculature.

MCCM Increases ICAM-1 and VCAM-1 mRNA in Human Fibroblasts  Northern blot analysis was performed to examine the effect of MCCM on ICAM-1 and VCAM-1 mRNA expression in cultured human dermal fibroblasts. Confluent human dermal fibroblasts were treated with MCCM for 1, 4, and 24 h or with control media containing calcium ionophore alone. VCAM-1 mRNA was undetectable and ICAM-1 mRNA was barely detectable in the fibroblasts cultured in the control media (Fig 9). In contrast, incubation with MCCM increased the steady-state levels of ICAM-1 and VCAM-1 mRNA expression by 1 h. The maximal accumulation was observed at 4 h and decreased toward baseline by 24 h (Fig 9). No significant change in mRNA levels for GAPDH

Figure 5. MCCM-induced time course and dose response of adhesion molecule expression on fibroblasts. Cell surface expression of ICAM-1 and VCAM-1 on cultured human dermal fibroblasts (A), human synovial fibroblasts (B), and human lung fibroblasts (C) was determined by ELISA. Fibroblasts were grown to confluence on 96-well tissue culture plates and then stimulated for the above indicated intervals with MCCM at the dilution of 1:10 or with incremental amounts of MCCM for 24 h. The data represent the mean of four experiments in human dermal fibroblasts (A) and the mean of three experiments in human synovial fibroblasts (B) and human lung fibroblasts (C), all performed in triplicate (± SEM). Ctrl, control.

Figure 6. Flow cytometric analysis of ICAM-1 and VCAM-1 expression on human dermal fibroblasts induced by MCCM. Confluent human dermal fibroblasts were stimulated for 24 h with MCCM (1:10). Within each graph, the adhesion molecules as depicted were quantitated under identical conditions in the absence of MCCM (B) and the presence of MCCM (C).

Figure 7. Induction of ICAM-1 and VCAM-1 cell surface expression on human dermal fibroblasts by mast cell products. The products were added to the confluent fibroblasts in the concentrations as depicted for 24 h. The results represent the mean ± SEM of three experiments performed in triplicate.
control probe was found after MCCM treatment (Fig 9, bottom), confirming that the differences noted above were not due to mRNA loading, transfer, or nonspecific stimulation. Densitometry was performed to normalize for slight variations in GAPDH bands on the Northern blots (Table 1), thus confirming the above interpretation.

**Mast Cell Degranulation Induces Expression of ICAM-1 in a Skin-Equivalent Model** Because the prior studies were all performed using monolayer cell cultures, mast cell degranulation in situ was induced in a skin-equivalent model to determine whether fibroblasts and/or keratinocytes respond in a similar fashion while in a three-dimensional organ culture system. Mast cell degranulation was accomplished by addition of calcium ionophore, as described in the in vitro studies. After overnight incubation, the specimens were snap-frozen for immunohistochemical processing to assess ICAM-1 and VCAM-1 expression. Frozen sections were stained with anti-ICAM-1 and VCAM-1 MoAbs, as described in Materials and Methods. As shown in Fig 10B, marked up-regulation of ICAM-1 was observed after mast cell degranulation. Intense staining was seen within dermal fibroblasts and in groups of keratinocytes. Prominent basal epidermal staining was observed in both control cultures (i.e., lacking mast cells and with mast cells but with no added calcium ionophore A23187; Fig 10A) and experimental cultures (i.e., with mast cells and calcium ionophore A23187 added; Fig 10B). Essentially no increase in VCAM-1 staining was observed after mast cell stimulation. Cultures without mast cells revealed no alterations in ICAM-1 or VCAM-1 after calcium ionophore treatment.

**DISCUSSION**

Our studies clearly document that mast cell products can stimulate T-cell adhesion to fibroblasts. The enhanced T-cell adhesion appears to be mediated by up-regulating ICAM-1 and VCAM-1 on fibroblasts. Induction of these adhesion molecules was observed when mast cells were cocultured with fibroblasts and subsequently degranulated. Resting mast cells attach to fibroblasts without inducing adhesion molecule expression. However, direct mast cell contact was not required, as MCCM was also effective in stimulating adhesion molecule expression on fibroblasts. Nonetheless, heterotypic coculture experiments demonstrated that a ratio of one stimulated mast cell per 100 fibroblasts was sufficient to increase expression of ICAM-1 and VCAM-1 significantly. The expression resulted from mRNA induction, which was detectable by 1 h. Surface protein expression followed within 4 h, peaked at 24 h, and decreased slightly toward baseline by 48 h, especially VCAM-1 expression. Our studies also indicated that ICAM-1 induction results from TNF-α release by mast cells. Induction of VCAM-1 is more complex and other mediators are presumably involved, although TNF-α is nonetheless an important contributing factor (Fig 8). It is conceivable that cofactor(s) are released from the mast cells that synergize with TNF-α or in some manner amplify its effect. This remains to be defined further in future studies, especially those using human mast-cell–derived products.

In the evolution of a local inflammatory reaction, circulating leukocytes transmigrate endothelial cell barriers into the extravascular space and accumulate by mechanisms that remain to be elucidated fully. The retention of leukocytes in tissue compartments is partly regulated by integrins on trafficking leukocytes binding to subendothelial matrix substances (i.e., cell-matrix interactions), and partly by interactions of surface adhesion molecules on cells (such as fibroblasts and epithelial cells) residing within extravascular tissue (i.e., cell-cell interactions). We speculated that degranulating mast cells might release products that stimulate cell-cell interactions by inducing adhesion molecule expression. Previous data from our laboratory, as well as others, have suggested that degranulating mast cells up-regulates endothelial cell surface molecules, including E-selectin, ICAM-1, and VCAM-1 [4,14,15]. The capacity of mast cells to mediate adhesion molecules on extravascular cells was initially suggested by Ioffreda et al [16] by demonstrating redistribution of α6 integrin on basal cell keratinocytes and up-regulation of α6β1 and α6β4 integrins on Langerhans cells after mast cell degranulation.

It is widely appreciated that adhesion molecule expression is regulated by cytokines [17,18], including TNF-α, IL-1, and IL-4 [9]. Because mast cells are capable of synthesizing a number of cytokines, including TNF-α, IL-1, IL-4, transforming growth factor-β, and IL-6 [19], it seemed plausible that mast-cell–derived cytokines were largely responsible for ICAM-1 and VCAM-1 expression on fibroblasts. Our studies confirmed this to a certain degree. Neutralizing antibody to TNF-α essentially abrogated up-regulation of ICAM-1. However, VCAM-1 expression was only partially suppressed, suggesting that other mast cell products were also important in VCAM-1 induction. These studies must be interpreted with caution, however, because cross-species of cells were required and murine IL-1β or IL-4, for example, may not bind effectively to human cells. Nonetheless, direct incubation of fibroblasts with histamine or heparin failed to show significant effects. Further studies will be required to define any other active sub-

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**Figure 8. Inhibition of MCCM regulatory effect on ICAM-1 and VCAM-1 expression.** The adhesion molecule expression on human dermal fibroblasts was determined by ELISA at 24 h after preincubation of neutralizing antibodies (10 ng/ml) to MCCM as indicated. Data represent the mean ± SEM of two experiments performed in triplicate wells. Ctrl, control.

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**Figure 9. Induction of ICAM-1 and VCAM-1 mRNA in human dermal fibroblasts by MCCM.** Confluent human dermal fibroblasts were stimulated by MCCM for the time intervals indicated, followed by Northern hybridization for ICAM-1 (A) and VCAM-1 (B) mRNA (top). Re-probing the membrane for GAPDH cDNA (bottom) did not reveal any differences over this time in response to MCCM.
stance(s) that mast cells secrete that regulate fibroblast adhesiveness. The finding that TNF-α was important in these studies is consistent with previous reports using tissue explants ex vivo, in which degranulated mast cells up-regulated E-selectin dermal microvascular endothelial cell expression [14,20]. In the dermis, mast cells appear to store TNF-α and serve as a major repository for this potent cytokine [20]. TNF-α, regardless of its source, also serves to prolong the survival of inflammatory cells, thus delaying programmed cell death [21]. Therefore, mast-cell-derived mediators, such as TNF-α, induce inflammatory responses by multiple pathways: stimulating transmigration via activation of endothelial cells, promoting leukocyte retention by up-regulating fibroblast adhesiveness, and prolonging the survival of inflammatory cells at perivascular sites.

We used a skin-equivalent organ culture system to assess ICAM-1 and VCAM-1 expression after mast cell degranulation, as our previous experience with skin explants indicated intense staining along the microvasculature precluding examination of extravascular expression. Using the skin-equivalent organ culture system, we were able to monitor adhesion molecule expression in the absence of endothelial cells and associated vascular structures. In this system, ICAM-1 expression was markedly up-regulated by mast cells, displayed within the epidermis as well as on individual fibroblasts. VCAM-1 expression was not detected after mast cell stimulation, suggesting several possibilities. Conceivably, VCAM-1 regulation differs when fibroblasts are placed in three-dimensional matrices rather than cultured as monolayers on plastic. The three-dimensional dermal matrix may not permit free diffusion of mast-cell-derived factors, thus preventing the responsible mediator from reaching target fibroblasts. Further studies are warranted to investigate these possibilities and to confirm this potentially important observation. In separate studies using human foreskins in which mast cells were stimulated in situ, we have observed similar findings, indicating more prominent ICAM-1 than VCAM-1 expression.

Although not the main focus of our study, the up-regulation of keratinocyte adhesion molecules within the epidermis of this skin culture system was unexpected and suggests yet another mechanism for lymphocyte retention at an inflammatory site [22]. Cytokines such as TNF-α are known to up-regulate keratinocyte ICAM-1 expression in vitro [23]. The role of mast cells in stimulating epidermal cells, however, has received minimal attention in the past.

In summary, our experimental results suggest a potential mechanism by which mast cells facilitate an inflammatory reaction, as observed in studies using mast-cell-deficient animals. In immune-complex-mediated peritonitis, the absence of mast cells greatly dampened the intensity of neutrophil extravasation [24], similar to that observed with a model of cutaneous acute inflammation [25]. In these studies, the mechanisms underlying the requirement for mast cells were not probed other than to suggest a role for TNF-α. Our studies indicate that mast cell products act on microvascular endothelial cells [4] as well as extravascular fibroblasts to incite and sustain an inflammatory response. In this regard, it is interesting that Heard et al. [26] performed ultrastructural analyses and noted mast cells often co-occur with fibroblasts, occasionally associated with lymphocytes under normal conditions. In allergic conditions, increased expression of adhesion molecules in situ has been well documented [27,28]. In fact, these observations have provided a mechanistic framework for considering therapeutic intervention using MoAbs to specific adhesion molecules [29]. Future studies will provide a better understanding of the precise role that mast cells play in inflammatory processes.

Figure 10. Photomicrograph of skin equivalent stained for ICAM-1 expression. Mast cells were cocultured with dermal fibroblasts in the organotypic culture at a ratio of 1:4. Calcium ionophore (500 ng/ml) was added to the skin equivalent containing mast cells (B) and in the absence of mast cells as control (A). Degranulation of mast cells induced marked up-regulation of ICAM-1 within dermal fibroblasts and in groups of keratinocytes (B). Bar, 240 μm.
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