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Signal Transduction via CD43 (Leukosialin, Sialophorin) and Associated Biological Effects in a Human Mast Cell Line (HMC-1)

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CD43 has been shown to be involved in the regulation of cellular adhesion and activation of leukocytes, but its functional significance for mast cell biology has been poorly defined. We demonstrate here that mAb engagement of surface CD43 on human leukemic (HMC-1) mast cells initiates a signaling cascade which involves protein kinase C, while tyrosine kinases appear to play a minor role, as evidenced by effects of different kinase inhibitors on homotypic aggregation induced via CD43. Furthermore, administration of an activating anti-CD43 mAb is shown to induce and promote TNF-α- and to enhance IL-8-secretion from HMC-1 cells, but it does not initiate histamine, tryptase, or LTC_4 release, suggesting that the intracellular pathways leading to aggregation and release of certain mast cell mediators are differentially regulated. Additionally, engagement of CD43 on HMC-1 cells leads to down-regulation of CD43 surface expression, implying that CD43 may be potentially involved in its own regulation.

Mast cells are specialized effector cells with key functions in a variety of physiological and pathological processes (reviewed in 1, 2). Although their mature forms reside exclusively within tissues, their progenitors are derived from $CD34^+$ hematopoietic stem cells (3) which enter the tissue where they differentiate under the influence of environmental growth factors. So far, very little is understood about the maturation stage of extravasating mast cell precursors as well as about the basic mechanisms underlying the process of diapedesis. However, studies on the extravasation of a variety of leukocytes have revealed striking similarities between different cell types and established a key role for adhesion receptors in this process (4, 5). In this regard, evidence is accumulating that regulated adhesive cell-cell interactions are also involved in the process of mast cell precursor extravasation (6, 7). Furthermore, adhesion receptors expressed on the surface of mast cells are thought to mediate a variety of interactions between these cells and other tissue or inflammatory cells, e.g. during mast cell mediated antigen presentation (8), and/or with components of the extracellular matrix (9, 10). Regulation of cellular adhesion may be accomplished by a variety of soluble stimuli as well as by cross-linking of certain cell-surface receptors.

A significant role in the regulation of cellular adhesion has been attributed to CD43 (leukosialin, sialophorin), the major sialoglycoprotein of circulating leukocytes (11). In this regard, monoclonal antibodies directed against CD43 have been shown to mediate intracellular signals leading to homotypic cell aggregation as well as to enhanced cellular affinity for different substrates (12–18).

Although CD43 has been reported to be constitutively expressed by cells of the human mast cell line HMC-1 as well as by mature human mast cells localized at different anatomical sites (18–20), its role in mast cell activation has not been established yet. Previous findings from our laboratory, however, presented evidence that mAbs to CD43 are capable of inducing homotypic aggregation of HMC-1 cells (18).

In the present investigation, we sought to clarify a possible involvement of CD43 in the activation of human mast cells and to shed light on the underlying signaling mechanisms. We report that anti-CD43 mediated activation leading to aggregation of HMC-1 cells involves a signal transduction cascade which depends upon protein kinase C while participation of tyrosine kinases appears to be of only minor significance. Furthermore, we demonstrate a functional role of CD43 also in the regulation of its own surface expression and on cytokine secretion from HMC-1 cells. On the other hand, CD43 is apparently not implicated in the release of preformed or newly synthesized mast cell mediators, such as histamine, tryptase, or LTC_4.

MATERIALS AND METHODS

Materials. Phorbol-12-myristate-13-acetate (PMA), cyclohexiimide, actinomycin D, cytochalasin B, staurosporine, N, N-dimethyls-

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phingosine, genistein, and 2-deoxy-D-glucose were purchased from Sigma (München, Germany).

Bisindoylmaleimide I, Ro 31-8220, a myristoylated EGF-R fragment, and tyrphostin 25 were supplied by Calbiochem (San Diego, CA). The monoclonal antibody to CD43 DF-T1 (azide-free preparation) and FITC-labeled DF-T1 were from Serva (Heidelberg, Germany). FITC-conjugated anti-glycophorin A mAb (clone D2.10) was purchased from Immunotech (Marseille, France), and mAb CAL3.34 (directed against human ICAM-3) from R&D Systems (Wiesbaden, Germany). The isotype of all mAbs was mouse IgG_1.

Cell culture. The human leukemic mast cell line HMC-1 was kindly provided by Dr. Butterfield (21). The subclone 5C6, raised from the original cell line by limiting dilution, has been described (22). For all experiments, cells of the more differentiated 5C6 subclone were used. They were kept routinely in basal Iscove's medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, antibiotics (all from Seromed, Berlin, Germany), and 10^{-5} M monothioglycerol (Sigma).

Aggregation assay. Quantitative aggregation assays were performed essentially as described (23), with slight modifications. In brief, HMC-1 cells were plated in 96-well microtiter plates (Greiner, Frickenhausen, Germany) at 1×10^6/ml, preincubated at 37°C for 20 min with various inhibitors at the concentrations indicated and subsequently stimulated with the anti-CD43 mAb DF-T1 at 10 μg/ml at 37°C in a total reaction volume of 100 μl for 1 h or 24 h, respectively.

Suspensions of each sample were mixed by pipetting up and down 20 times and subsequently transferred to a Neubauer hemocytometer where the number of free cells and total cell number were assessed. Percentage of aggregation was determined as (1-number of free cells/total cell number) $\times$ 100.

Flow cytometry. HMC-1 cells at 1×10^6/ml were stimulated with the anti-CD43 mAb DF-T1 at 1 μg/ml for the times indicated, washed twice in Ca^{2+}/Mg^{2+}-free PBS, incubated with diluted AB-serum (Biotest, Dreieich, Germany) for 30 min at 4°C, washed and reacted with FITC-labeled DF-T1 (20 μl per sample, 30 min at 4°C). Control cells were kept at 37°C in the absence of any stimulus and processed in the same manner.

After washing, cells were fixed with formaline and analyzed by an EPICS XL flow cytometer (Coulter Electronics, Krefeld, Germany). At least 5000 cells were investigated per sample.

Mean channel fluorescence (MCF) was considered to correlate roughly with cell surface molecule density. For negative control, cells were stained with the irrelevant mAb D2.10, and its MCF never exceeded the value of 0.5.

Measurement of mast cell mediator release. The activity of tryptase was detected according to Harvima et al. (24), with slight modifications. In brief, 50 μl of dialysed supernatants of stimulated or unstimulated HMC-1 cells were mixed with Tris-HCl buffer (150 mM, pH 7.6) containing α1-antitrypsin and heparin (both from Sigma) followed by addition of Z-Gly-Pro-Arg-pNitroanilid (Sigma). Extinction was measured at 405 nm using a Titertek Multiskan MCC/340 photometer.

Commercially available EIA-kits were used for detection of histamine (Immunotech, Marseille, France) and LTC_4 (Cayman Chemical Company, Ann Arbor, MI) in the supernatants of HMC-1 cells, following the manufacturer's instructions.

ELISA. HMC-1 cells (at 1×10^6/ml) under serum-free conditions were treated with the anti-CD43 mAb DF-T1, the anti-ICAM-3 mAb CAL3.34 (both at 10 μg/ml), or medium alone for 24 h at 37°C. In other experiments, HMC-1 cells were pretreated or not with DF-T1 or CAL3.34 (both at 10 μg/ml) for 1 h at 37°C prior to addition of PMA (25 ng/ml) and incubated for further 24 h at 37°C. After this period, supernatants were frozen in liquid nitrogen and kept at −80°C. The concentrations of interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) in cell-free supernatants were determined by using commercially available enzyme-linked immunosorbent assays (R&D Systems, Wiesbaden, Germany), as detailed by the supplier.

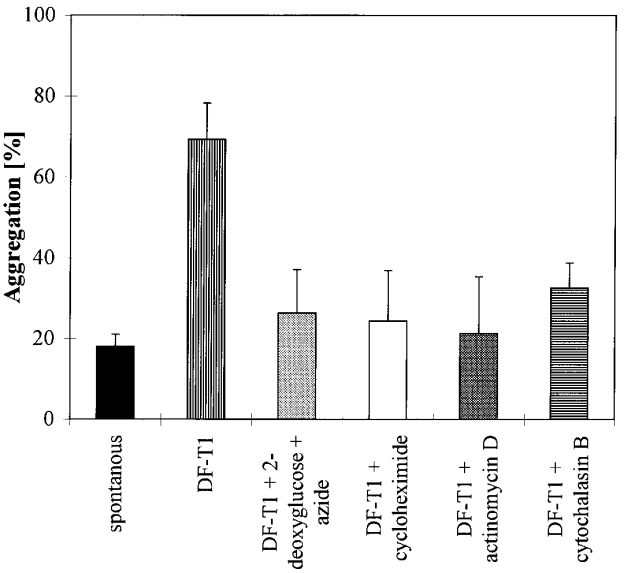


FIG. 1. Homotypic aggregation mediated via CD43 requires cellular metabolism, de novo protein synthesis, and a functional cytoskeleton. Inhibition of homotypic aggregation was studied in a quantitative assay. Cells at 1×10^6/ml were preincubated with 2-deoxyglucose + sodium azide (5 mM and 0.2%, respectively), cycloheximide (10 μg/ml), actinomycin D (10 μg/ml), or cytochalasin B (100 μM) for 20 min and subsequently stimulated with the anti-CD43 mAb DF-T1 (10 μg/ml). Aggregation was determined after 24 h of stimulation as described under Materials and Methods. Similar results were obtained after 1 h of treatment with the mAb, except that after 1 h, aggregation was virtually unaffected by either cycloheximide or actinomycin D. Data are the mean ± SD of at least six independent assays. Aggregation was reduced in a highly significant manner ($p < 0.001$) for all inhibitors tested, as determined by Student's unpaired *t*-test.

Statistics. Differences between values were compared by the two-tailed *t*-test according to Student.

RESULTS

Homotypic Aggregation of HMC-1 Cells Triggered via CD43 Depends upon Cellular Metabolism and a Functional Cytoskeleton

Using a quantitative aggregation assay, we first tested whether adhesion induced by the anti-CD43 mAb DF-T1 depended on normal cellular functions, such as active metabolism and a functional cytoskeleton. Aggregation was determined after 24 h of incubation with DF-T1. As presented in Fig. 1, aggregation was inhibited by metabolic depletion of cellular ATP by prior incubation with 2-deoxyglucose plus sodium azide. Moreover, paralysis of the cytoskeleton by cytochalasin B strongly inhibited anti-CD43 induced aggregation, implying that aggregation was not the result of mere cross-bridging of cells by the mAb applied. Inter-

estingly, aggregation assessed after 24 h was strongly affected by either cycloheximide or actinomycin D (Fig. 1). Cell viability was not reduced after the incubation period with either inhibitor, as evidenced by trypan blue exclusion (data not shown). In contrast, when aggregation was determined after 1 h, no inhibition by either cycloheximide or actinomycin D could be detected (data not shown), suggesting that de novo protein synthesis was not required for induction, but well for maintenance of HMC-1 cell adhesion induced via CD43.

Involvement of Protein Kinase C in Anti-CD43 Mediated Homotypic Adhesion

The intracellular signal transduction pathways responsible for anti-CD43 induced homotypic aggregation of HMC-1 cells were further elucidated by examining the effects of various protein kinase inhibitors on the adhesive response.

Since activation of protein kinase C (PKC) via CD43 has already been reported for human monocytes (25), we first employed several inhibitors primarily affecting PKC action. As shown in Fig. 2 A-E, all of the PKC inhibitors tested (staurosporine, N, N-dimethylsphingosine, bisindoylmaleimide I, Ro 31-8220, and myristoylated EGF-R fragment) were effective at inhibiting HMC-1 cell adhesion in a dose-dependent manner. On the other hand, the tyrosine kinase inhibitor genistein was found to affect adhesion only at rather high concentrations (75 μM) in a statistically significant fashion (Fig. 2, F). At this concentration, genistein may also act on other kinases such as PKC.

To further clarify a possible implication of tyrosine kinases in CD43-triggered signaling, the effect of tyrphostin 25, a more selective inhibitor of tyrosine kinases, was studied. This compound produced a slight and dose-dependent inhibition of HMC-1 aggregation which, however, did not reach statistical significance up to a dose of 25 μM (not shown).

These collective data indicate that engagement of CD43 initiates a signal transduction cascade that may involve PKC and tyrosine kinases. While PKC action appears to be of pivotal relevance to CD43-mediated homotypic HMC-1 aggregation, however, participation of tyrosine kinases appears to be of minor significance.

CD43 Is Transiently Down-regulated on HMC-1 Cells Following Stimulation via CD43

CD43 is known to be down-regulated on activated cells (14, 26–30). We were therefore interested in possible effects of the stimulating anti-CD43 mAb DF-T1 on HMC-1 surface expression of CD43.

To this end, kinetic studies were performed in which HMC-1 cells were stimulated with DF-T1 at 1 μg/ml for 15 min up to 24 h, and the remaining CD43 binding sites were determined thereafter by staining cells using FITC-conjugated anti-CD43-mAb and analyzing CD43 expression by flow-cytometry. As presented in Fig. 3, a transient down-regulation of CD43 surface density, as evidenced by reduced mAb binding capacity, could clearly be observed in six independent assays, reaching a minimum after 2–6 h of incubation with DF-T1. CD43 was subsequently up-regulated, reaching its basal level after 24 h of treatment. Surface expression of ICAM-1, used as a control, remained unchanged over the observation period upon stimulation of HMC-1 cells with DF-T1 (results not shown).

Signal Transduction via CD43 Does Not Lead to Release of Histamine, Tryptase, or LTC_4

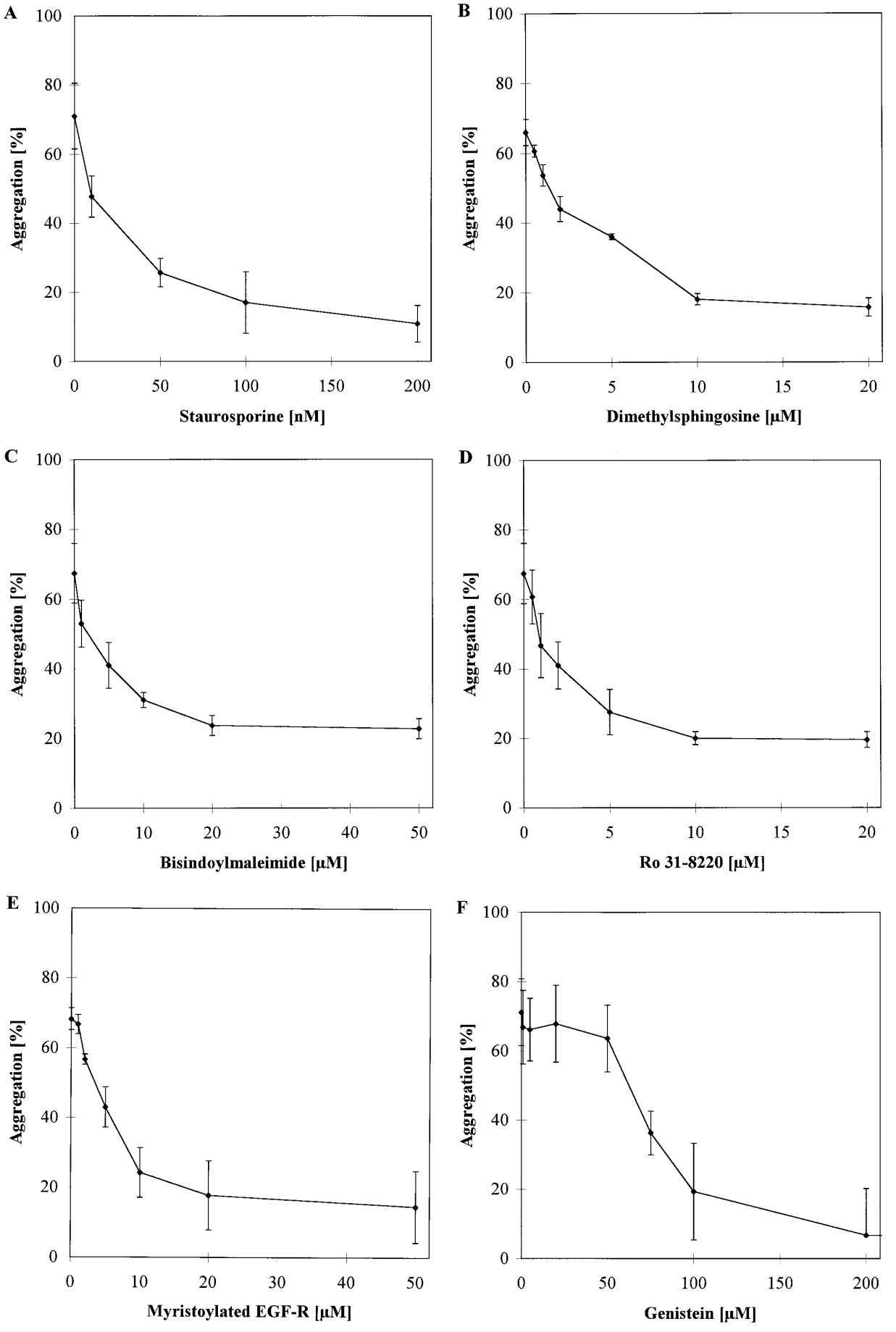
Release of preformed or newly synthesized mast cell mediators is a well established parameter for mast cell activation (reviewed in 2). We therefore studied the effects of the activating anti-CD43 mAb DF-T1 on mast cell secretion, as assessed by the measurement of histamine, tryptase, and LTC_4.

As demonstrated in Table 1, no appreciable increase in mast cell mediator secretion after a 30 min stimulation with DF-T1 could be observed, as compared to baseline (spontaneous) mediator release. Even prolonged incubation with anti-CD43 mAb of up to 4 h did not induce significant mediator release from HMC-1 cells (data not shown).

CD43-Mediated Signaling Modulates Secretion of TNF-α and IL-8 from HMC-1 Cells

Human mast cells are known to produce and release a wide array of cytokines upon appropriate stimulation (for review see 31 and references therein) among which mast cell-derived TNF-α and IL-8 play a significant role in modulating inflammatory and immune functions, especially by attracting and activating neutrophils during bacterial infections (32).

Since activation of human lymphocytes through CD43 has been reported to result in increased IL-2 secretion (12), we were interested whether CD43 mediated signaling may also modulate secretion of cytokines produced by human mast cells. To this end, HMC-1 cells were pretreated with the inducing mAb DF-T1 and subsequently exposed to PMA. This order of treatments was chosen since CD43 has been previously demonstrated to be down-regulated on HMC-1 cells following PMA treatment (30). As shown in Fig. 4A, stimulation of HMC-1 cells by DF-T1 alone resulted in an increase in TNF-α concentration in the supernatant of HMC-1 cells, as compared to baseline concentration of this cytokine. Accordingly, DF-T1 synergistically enhanced PMA-mediated TNF-α production (Fig. 4B) in a statistically significant fashion. IL-8 which is produced by HMC-1 cells only upon stimulation (33) could not be detected in supernatants of HMC-1 cells exposed to medium or DF-T1 alone (not illustrated). However,


A
Aggregation [%]
Staurosporine [nM]
B
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Dimethylsphingosine [µM]
C
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Bisindoylmaleimide [µM]
D
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Ro 31-8220 [µM]
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Myristoylated EGF-R [µM]
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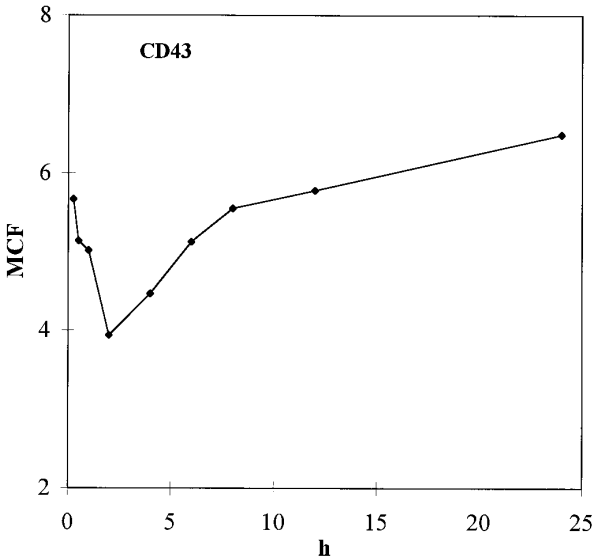

FIG. 3. Time-course of DF-T1-mediated modulation of CD43 on HMC-1 cells. HMC-1 cells at 1×10^6/ml were incubated with DF-T1 (1 μg/ml) for the times indicated. The remaining unoccupied CD43 surface molecules were detected by FITC-conjugated DF-T1 and flow-cytometry. Mean channel fluorescence (MCF) was considered to correlate roughly with surface molecule density. One representative experiment out of six is shown. The MCF value of untreated HMC-1 cells (corresponding to the entire CD43 surface density) in the experiment shown was 8.9. MCF - Mean Channel Fluorescence.

TABLE 1

Mast Cell Mediator Release after Stimulation of HMC-1 Cells with DF-T1

Mediator	n	Control	DF-T1
Histamine	2	100	103
Tryptase	2	100	102
LTC_4	4	100	108

HMC-1 cells at 1×10^6/ml were stimulated with the anti-CD43 mAb DF-T1 at 1.25 μg/ml for 30 min at 37° C. Control cells were kept in medium alone. Subsequently supernatants were analyzed for the presence of mast cell mediators by using EIA-kits for histamine and LTC_4, or by measuring the extinction at 405 nm using Z-Gly-Pro-Arg-pNA as a substrate for tryptase. Data are the mean of n independent experiments and are expressed as % of unstimulated control.

DF-T1 was able to enhance PMA-induced IL-8 secretion from HMC-1 cells in a highly significant manner (Fig. 4C). In contrast, an isotype-matched mAb to ICAM-3 (CAL3.34) which is abundantly expressed by human mast cells (manuscript in preparation) displayed no effects on spontaneous or PMA-induced secretion of either TNF-α or IL-8 (Fig. 4).

DISCUSSION

The present data indicate that CD43, expressed on the surface of human mast cells, is capable of mediating intracellular signals, as has been shown for other blood cells such as lymphocytes, neutrophils and monocytes. However, remarkable differences have been reported between distinct leukocyte species, with regard to the precise biological processes induced via CD43 as well as in the underlying signaling mechanisms. Specifically, anti-CD43 mAbs are known to induce homotypic aggregation and activation of mononuclear cells, as judged by increased hydrogen-peroxide production, when anti-CD43 mAb was combined with PMA (13), while enhanced proliferation, pronounced IL-2 secretion and induction of activation related antigens were reported for human lymphocytes upon their stimulation via CD43 (12, 34). Silverman et al. provided evidence that activation of peripheral blood mononuclear cells via CD43 initiates the generation of phosphoinositide-derived second messengers, Ca^{2+} mobilization and PKC activation which has been shown to be of greater significance for monocytes than for T cells (25). Neutrophils, on the other hand, have also been demonstrated to be aggregated by anti-CD43 mAb, but neither respiratory burst activity nor a significant rise in intracellular calcium or actin polymerization were observed (14). Rosenkranz et al. showed an involvement of tyrosine kinases but not PKC in the signal transduction cascade leading to homotypic aggregation upon cross-linking of CD43 on the neutrophil surface (16). The fact that signaling through CD43 in human mast cells involves PKC, as shown in this study, is in accordance with the hypothesis according to which mast cells and monocytes may share a common differentiation pathway (2, 31). While our present data suggest that tyrosine kinases may partly be involved in CD43-mediated signaling in HMC-1 cells, protein tyrosine phosphorylations are most likely of minor importance as compared to PKC action. In addition to homotypic adhesion, we were interested whether engagement of CD43 on human mast cells may modulate other functions specifically implicated in mast cell activation, such as stimulus-dependent and

FIG. 2. Inhibition of anti-CD43-induced homotypic aggregation by protein kinase inhibitors. HMC-1 cells were incubated for 20 min at 37 °C with varying concentrations of staurosporine (A), dimethylsphingosine (B), bisindoylmaleimide (C), Ro 31-8220 (D), a myristoylated EGF-R peptide (E), or genistein (F) prior to stimulation with the anti-CD43 mAb DF-T1 (10 μg/ml). Percentage of aggregation was determined after 24 h as described in Materials and Methods. Very similar results were obtained after 1 h of incubation. Results are the mean ± SD of at least four independent tests. Aggregation was reduced in a highly significant manner ($p < 0.001$) at concentrations equal or greater 10 nM (staurosporine), 5 μM (dimethylsphingosine, bisindoylmaleimide, Ro 31-8220, myristoylated EGF-R peptide), or 75 μM (genistein), as determined by Student's unpaired *t*-test.

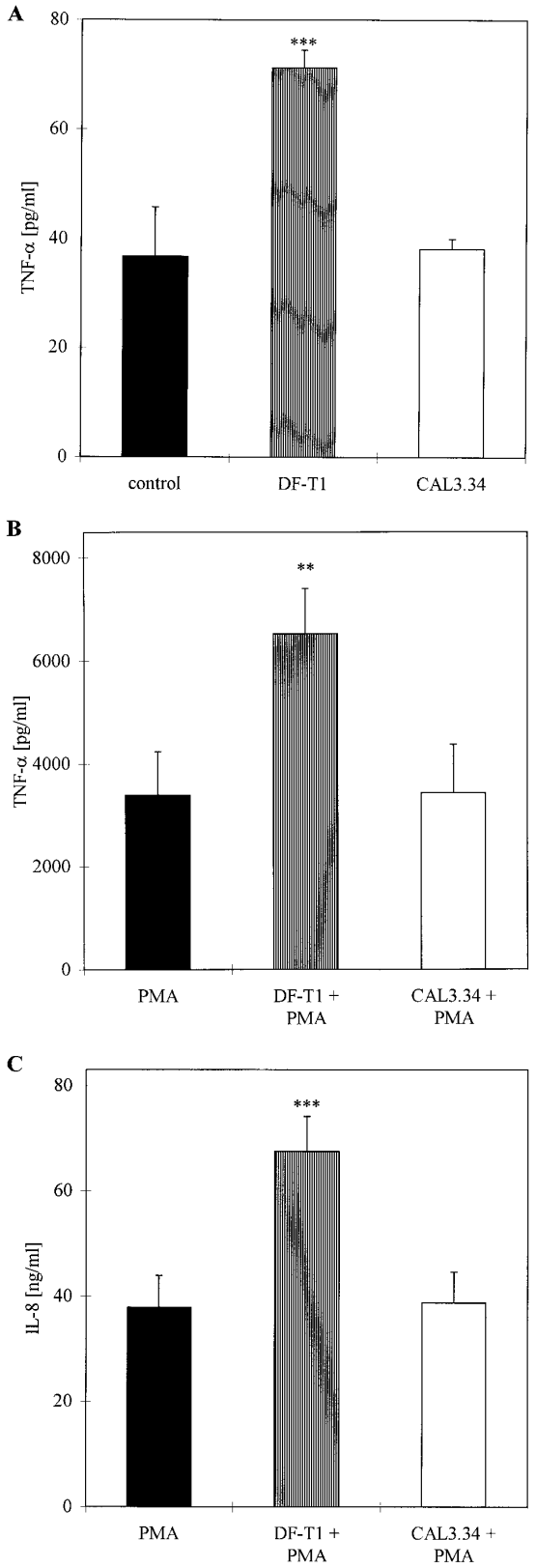

FIG. 4. Modulation of cytokine release upon HMC-1 cell activation via CD43. A. Cultures of HMC-1 cells (1×10^6/ml) under serum-free conditions were treated with either anti-CD43 mAb DF-T1 at 10 μg/ml, anti-ICAM-3 mAb CAL3.34 at the same concentration, or medium alone (control) for 24 h at 37°C. Levels of TNF-α (expressed as pg/ml) in culture supernatants from HMC-1 cells were determined by ELISA using three separate cell preparations and are expressed as mean ± SD. B and C. Cultures of HMC-1 cells (1×10^6/ml) under serum-free conditions were pretreated with either DF-T1 (10 μg/ml), CAL3.34 (10 μg/ml), or medium alone for 1 h at 37° C prior to addition of PMA and incubation for 24 h at 37° C. Levels of TNF-α (expressed as pg/ml) (B) and IL-8 (expressed as ng/ml) (C) were determined by ELISA, as described above. **$p < 0.01$, ***$p < 0.001$ versus the corresponding control (medium or PMA-treated cells).

spontaneous mast cell mediator release and cytokine production. While there was no significant induction of immediate type mediator secretion, an increase in the release of TNF-α and IL-8 from HMC-1 cells was observed when cells were pretreated with the anti-CD43 mAb DF-T1 with even an induction of TNF-α secretion by DF-T1 alone, thus distinguishing mast cell activation through CD43 from most other known activation pathways. This finding, however, fits the concept that cytokine secretion by mast cells is not necessarily preceded by histamine release (35, 36) and that release of diverse cytokines from mast cells is differentially regulated (37).

A further aspect in response to anti-CD43 mediated cell activation is down-regulation of CD43. Down-modulation of CD43 as a result of cross-linking of CD43 on the surface of lymphocytes and granulocytes has been reported before (28). Furthermore, stimulation of neutrophils and HMC-1 cells with PMA is also known to result in a decrease in CD43 surface expression, most probably due to a proteolytic shedding mechanism (14, 26–30). Down-regulation of CD43 is generally believed to be linked to cell activation. This hypothesis has been based on the findings that CD43 is capable of providing a repulsive barrier around cells which results in diminished cell-cell-interactions (38–40). An important role of CD43 in controlling mast cell adhesion is also anticipated from our recent finding where enhanced expression of CD43 in response to retinoic acids could be correlated to altered adhesive behavior of HMC-1 cells (23). We here provide evidence that mAb occupation of surface CD43 on HMC-1 cells, most likely mimicking the effects of a so far unidentified natural ligand(s), leads to down-regulation of the CD43 glycoprotein itself, suggesting that this receptor is inherently involved in its own regulation.

The discrepancy between mediator release and aggregation of mast cells is of great significance, clearly demonstrating that the intracellular pathways leading to degranulation are strictly distinct from those leading to homotypic aggregation. Changes in the adhesive phenotype of mast cells might be responsible for their extravasation, for interaction of mast cells with immune and other cells in the tissue, and for fine-tuning of cytokine production during an inflammatory response, whereas broad range mediator secretion would

only occur in tissue in response to immunological or inflammatory signals. The cell would thus be able to distinguish between different stimuli by displaying different activating responses depending on the precise necessities given by the environmental circumstances. These results show parallels to those reported for basophils where aggregation and mediator release have been demonstrated to represent uncorrelated processes which may occur in response to different stimuli (41).

Taken together, our results demonstrate that mast cells, like other leukocytes, may be activated through CD43, while the exact stimulatory response depends on the cell type studied. This mechanism of mast cell activation appears to be highly selective in that only certain mast cell related functions are affected by signaling via CD43, while others are not.

There might be differences in the signaling properties between immature leukemic (HMC-1) and differentiated mast cells of different organs. The fact that CD43 is abundantly expressed also on mature human mast cells from lung, gut, uterus, and skin (19, 20, and own unpublished observations), however, combined with the observation that CD43 expression is obviously regulated on human skin mast cells *in vitro* (23) and *in vivo* in the context of certain inflammatory skin diseases (manuscript in preparation), strongly implies an important role for this receptor in mast cell physiology under both normal and pathological conditions.

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