Selective Regulation of RNK-16 Cell Matrix Metalloproteinases by the EP_4 Subtype of Prostaglandin E_2 Receptor†

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ABSTRACT: Cell surface expression of multiple structurally and functionally distinct prostaglandin E_2 (PGE_2) receptors (Rs), designated the EP_1, EP_2, EP_3, and EP_4 Rs, is a principal determinant of the diverse cellular effects of PGE_2. The RNK-16 line of rat large granular lymphocytes, which has served as a model for natural killer cells, coexpresses a mean of 1092 EP_3 Rs and EP_4 Rs per cell with a mean K_d of 2.7 nM. The presence of the EP_3 and EP_4 Rs and the absence of the EP_1 and EP_2 Rs were revealed by inhibition of [^{3}H]PGE_2 binding by the EP_3/EP_1R agonist sulprostone, the EP_3/EP_2/EP_4R agonist M&B 28767, and the EP_2/EP_4/EP_3R agonist misoprostol but not by the EP_1R antagonist SC-19220 or the EP_2R agonist butaprost. Functional EP_4 R expression was confirmed by finding that PGE_2 and misoprostol, but not butaprost or sulprostone, evoked increases in the intracellular concentration of cyclic AMP ($[cAMP]_i$) in RNK-16 cells. Matrix metalloproteinase (MMP)-1 and -3 were identified by zymography and Western blots as the principal MMPs secreted by RNK-16 cells. Secretion of both MMPs by RNK-16 cells attained a maximal level after 24 h of incubation and was enhanced significantly by 10^{-9} to 10^{-7} M PGE_2, 10^{-6} M misoprostol, and 10^{-4} M dibutyryl cyclic AMP, but not by the EP_3R agonist sulprostone. Thus, the effect of PGE_2 on RNK-16 cell MMP secretion is mediated by an EP_4 R-dependent mechanism involving increases in $[cAMP]_i$. The migration of RNK-16 cells across micropore filters, without or with a layer of Matrigel, was stimulated chemokinetically by PGE_2 and misoprostol. PGE_2-elicited chemokinesis of RNK-16 cells across a Matrigel model basement membrane, but not across a microfilter alone, was suppressed by the GM 6001 inhibitor of MMP activities. Stimulation of MMP activities in RNK-16 cells by the EP_4R thus facilitates migration of the NK cells across vascular basement membranes.

Prostaglandin E_2 (PGE_2)[1] is a potent mediator of many biological functions in the cardiovascular, pulmonary, renal, endocrine, gastrointestinal, neural, reproductive, and immune systems (Coleman et al., 1994). Cell surface expression of multiple structurally and functionally distinct receptors (Rs) for prostaglandin E_2 (PGE_2) is a principal determinant of the diverse cellular effects of PGE_2. PGE_2 is recognized and transduces cellular effects specifically by interacting with PGE_2 Rs of at least four subtypes, designated the EP_1, EP_2, EP_3 and EP_4 Rs. These subtypes of PGE_2 Rs differ in structure, ligand-binding properties, tissue distribution, and coupling to signal transduction pathways (Coleman et al., 1994). The EP_1 Rs and EP_3 Rs mediate increases in the intracellular concentration of calcium ($[Ca^{2+}]_i$) (Coleman et al., 1994; An et al., 1994; Yang et al., 1994), whereas EP_2 Rs and EP_4 Rs transduce increases in intracellular concentration of cAMP ($[cAMP]_i$) and EP_3 Rs suppress increases in $[cAMP]_i$ that have been elevated by forskolin (Coleman et al., 1994; An et al., 1993, 1994).

Natural killer (NK) cells are a lymphoid population of lineage, morphology, and spontaneous cytotoxic functions distinct from T or B cells, which kill cells infected by virus or bacteria, as well as many transformed cell types (Trinchieri, 1989, 1994; Moretta et al., 1994). The cytotoxic principles secreted by NK cells include serine proteases with trypsin-like or chymotrypsin-like properties (Sayers et al., 1994; Hudig et al., 1993; Smyth et al., 1992). Less is known about other NK cell proteases or constituents that initiate and control their migration in connective tissues and their localization at sites of tumors and microbial invasion. PGE_2 has diverse effects on the differentiation and cytotoxic functions of NK cells (Vaillier et al., 1995; Lauzon & Lemaire, 1994; Baxevanis et al., 1993; Linnemeyer & Pollack, 1993). However, the representation and functions of the subtypes of PGE_2 Rs have not been characterized in NK cells. We now report that a cultured line of rat NK cells, termed RNK-16, coexpresses the EP_3 and EP_4 subtypes of PGE_2 Rs and that PGE_2 evokes a unique profile of matrix metalloproteinase (MMP) activities which are required for passage of RNK-16 cells through a Matrigel model of the vascular basement membrane. The activation of the MMPs is regulated by PGE_2 through an EP_4 R-dependent mechanism involving increases in $[cAMP]_i$.

MATERIALS AND METHODS

Materials. [5,6,8,11,12,14,15-^{3}H]PGE_2 (153 Ci/mmol, Dupont-New England Nuclear, Boston, MA), M&B 28767

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[1] Abbreviations: PGE_2, prostaglandin E_2; NK cell, natural killer cell; MMP, matrix metalloproteinase; R, receptor; IBMX, 3-isobutyl-1-methylxanthine; PMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NCAM, neurol cell adhesion molecule; LGL, large granular lymphocytes.

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(Rhone-Poulenc Rorer Research, Dagenham, Essex), sulprostone (Schering Pharmaceuticals, Berlin), PGE_1, PGD_2, $PGF_2\alpha$, PGI_2 (Upjohn Co., Kalamazoo, MI), SC 19220, misoprostol (Searle, Skokie, IL), a cAMP radioimmunoassay kit (Dupont-New England Nuclear), mouse monoclonal IgG antibodies specific for MMP-1, MMP-2, MMP-9 (Oncogene Science, Cambridge, MA), and MMP-3 (Bayer AG, West Haven, CT), 3-isobutyl-1-methylxanthine (IBMX), (*p*-amidinophenyl)methanesulfonyl fluoride (PMSF), Nutridoma (Boehringer-Mannheim, Indianapolis, IN), ovalbumin, forskolin, type A porcine skin-derived gelatin, bovine milk casein, and dibutyryl-cAMP (Sigma Chemical, St. Louis, MO) were obtained from the designated sources.

Culture of RNK-16 Cells. F344 rat leukemic LGL (large granular lymphocytes) cells of the RNK-16 line (Seaman et al., 1987) were cultured in RPMI 1640 (UCSF Cell Culture Facility) with 10% (v:v) FBS (Hyclone Laboratories, Logan, UT), 25 μM 2-mercaptoethanol, 100 u/mL penicillin G, and 100 μg/mL streptomycin (complete RPMI 1640). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air at a density of $(0.5-1.5) \times 10^6$ cells/mL by adding medium every 2−3 days.

Quantification of RNK-16 Cell Binding of [^{3}H]PGE_2 and cAMP Responses to PGE_2. Replicate suspensions of 1×10^6 RNK-16 cells in 100 μL of 100 mM NaCl−10 mM KH_2PO_4 (pH 6.0) with 1 mg/mL recrystallized ovalbumin were incubated with 3 nM [^{3}H]PGE_2 without and with 10^{-10} to 10^{-6} M nonradioactive PGE_2, other synthetic prostanoids, and agonists for 2 h at room temperature. Bound radioactivity was recovered by vacuum filtration (Hoeffer Scientific, San Francisco) of RNK-16 cells through 0.3% poly(ethyleneimine)-pretreated GF/C filters (Whatman), which were then washed with 12 mL of cold binding buffer for determination of radioactivity in an LC5801 scintillation counter. The binding of [^{3}H]LTB_4 (Amersham) was assessed similarly. Binding data were analyzed by the LIGAND computer program (Munson & Rodbard, 1980).

Identical suspensions of RNK-16 cells in 100 μL of Hanks' balanced salt solution with 1 mg/mL ovalbumin and 10 mM HEPES (pH 7.4) were preincubated with 1 mM IBMX, a cAMP phosphodiesterase inhibitor, for 10 min and incubated with PGE_2 and other agonists for 10 min at 37 °C. Ice-cold ethanol was added to a final concentration of 75%, and cAMP in 15000*g* supernatants was quantified by radioimmunoassay, according to the manufacturer's protocol.

Characterization of Matrix Metalloproteinases of RNK-16 Cells by Zymographic and Western Blot Analyses. FBS was found to interfere with the zymographic analyses in our studies. Thus, replicate pellets of 10^7 RNK-16 cells were washed three times with 40 mL of protein-free RPMI 1640 and incubated in 2 mL of protein-free 1:1 Iscove's−RPMI-1640 medium without and with 10^{-10} to 10^{-7} M PGE_2 and other eicosanoids at 37 °C in 5% CO_2 and 95% air for up to 24 h. The suspensions were then centrifuged at 15000*g* for 20 min at 4 °C, and replicate aliquots of the 15000*g* supernatants were electrophoresed to resolve secreted MMPs for quantification by enzymatic activity and immunoreactivity as described in the following procedures.

Thirty microliters of each supernatant, which represented the secretions from 1.5×10^5 RNK-16 cells, was electrophoresed in nonreducing 10% SDS−polyacrylamide gels, which had been copolymerized with 1 mg/mL type A porcine skin-derived gelatin or bovine milk casein as described (Leppert et al., 1995a), incubated in 2.5% Triton X-100 overnight at room temperature to remove SDS and in 50 mM Tris-HCl−50 mM NaCl−5 mM $CaCl_2$ for 48 h at 37 °C for protein digestion. After the undigested protein was stained with Coomassie blue, the change in staining of each band of protease activity was determined by densitometry with a Scan-Jet IIC and NIH Image 1.41 software, and the results were expressed as the relative percentage of untreated controls.

Replicate aliquots of the 15000*g* supernatants also were concentrated by approximately 50-fold with Centricon-10 concentrators (Amicon, Lexington, MA), and 5 μg of protein samples from each then was heated at 100 °C in 20 μL of 3% Laemmli's buffer prior to electrophoresis in 1 mm thick SDS−10% polyacrylamide gels at 100 V for 3 h at 4 °C along with rainbow prestained protein molecular weight standards (Amersham, Arlington Heights, IL). The proteins resolved were transferred by electroblotting to a 0.45 μm pore nitrocellulose membrane (Hybond, Amersham) at 100 V for 1 h. The blots were developed with 1 μg/mL mouse monoclonal IgG antibodies specific for MMP-1, MMP-2, MMP-3, and MMP-9, and then a 1:2000 dilution of horseradish peroxidase-labeled sheep anti-mouse IgG (Amersham), so that chemiluminescence analysis would permit densitometric quantification as described for zymography.

Assessment of Migration of RNK-16 Cells. Replicate suspensions of 0.8×10^6 washed RNK-16 cells in 0.2 mL of Iscove's medium with 50 u/mL penicillin G, 50 μg/mL streptomycin, and 2% (v:v) Nutridoma were added to the 6.5 mm diameter inserts of Transwell chambers (Costar, Cambridge, MA) over 8 μm pore polycarbonate filters (Nucleopore Corp., Pleasanton, CA) without or with a continuous even coating of 15 μL of Matrigel (Collaborative Research, Bedford, MA), which separated the cells from 500 μL of buffer in the lower compartment. A stimulus of 10^{-10} to 10^{-6} M PGE_2 or other prostanoid was present only in the lower compartment for chemotactic experiments and was added at the same concentration to the lower compartment and cell suspension for chemokinetic studies. The hydroxamic acid dipeptide analogue inhibitor of MMPs, HONH-$COCH_2$CH(i-Bu)CO-L-Trp-NHMe (GM6001), and the inactive peptide control L-Leu-L-Trp-methylamide-HCl (GM-2454) (Glycomed, Alameda, CA) were added to the lower compartment, cell suspension, and Matrigel layer at the same concentration of 10^{-8} to 10^{-5} M. Chambers with uncoated filters were incubated for the previously established optimal time of 4 h and those with Matrigel-coated filters for 18−60 h at 37 °C in 5% CO_2 in air. Inserts then were shaken for 10 min at room temperature to detach cells adhering to the bottom surface of the filters and the number of cells counted in the lower compartment. The migration responses were expressed as the total number of RNK-16 cells per mm^3 or, for some studies, as a percentage of the control value (100%) for migration in the absence of an inhibitor. In studies of the time course of migration, the magnitude of stimulation of chemokinesis through Matrigel by 10^{-8} M PGE_2 was a mean of 21% at 18 h and 146% at 54 h of the corresponding responses at 36 h (100%, $n = 2$), at which time all other studies were performed.

RESULTS

Coexpression of the EP_3 and EP_4 Rs by RNK-16 Cells. The specific binding of [^{3}H]PGE_2 to RNK-16 cells was

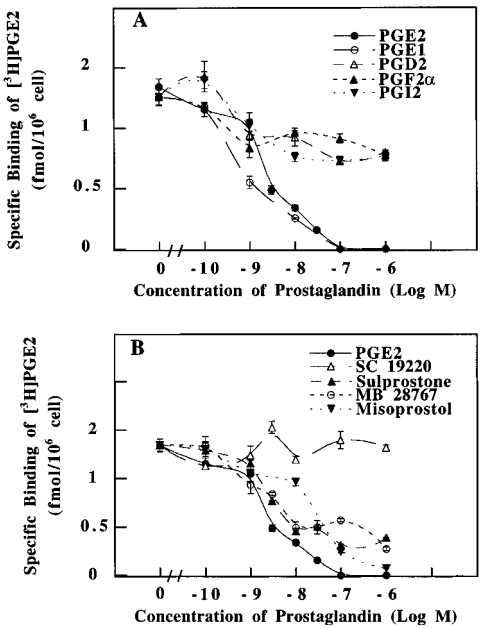

FIGURE 1: Specific binding of [^{3}H]PGE_2 to intact RNK-16 cells. Competitive inhibition of the binding of [^{3}H]PGE_2 by nonradioactive prostanoids (A) and synthetic prostaglandin analogues (B). Each point represents the mean ± SE of triplicate samples. The results shown are from one study that is representative of three experiments.

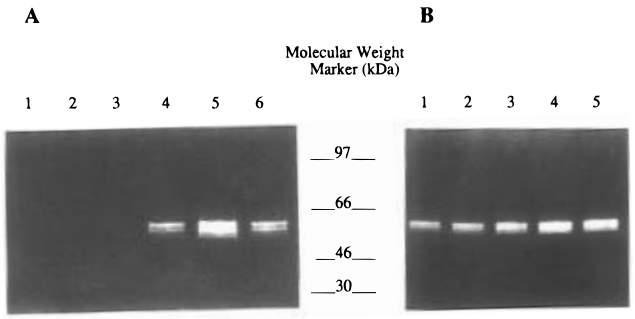

FIGURE 2: PGE_2 stimulation of secretion of MMPs by RNK-16 cells. (A) Time dependence. Supernatant samples in each lane are as follows: control at 4 h (lane 1) and 24 h (lane 4), 10^{-7} M PGE_2 treatment at 4 h (lane 2) and 24 h (lane 5), and 10^{-6} M LTB_4 treatment at 4 h (lane 3) and 24 h (lane 6). (B) Dose dependence. Supernatant samples in lanes 1−5 are treatment with 0, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-7} M PGE_2 for 24 h. Zymographic analyses of the proteins secreted from the same number of RNK-16 cells were performed as described. The result is representative of three experiments.

inhibited competitively by nonradioactive synthetic prostanoids with a rank order of potency of $PGE_2 = PGE_1 \gg PGF_{2\alpha} = PGD_2 = PGI_2$ (Figure 1A), whereas there was no detectable specific binding of [^{3}H]LTB_4 to RNK-16 cells. The binding of [^{3}H]PGE_2 to RNK-16 cells was characterized by a K_d of 2.7 ± 0.3 nM and a B_{max} of 1.8 ± 0.5 fmol/10^6 cells (1092 ± 302 PGE_2 Rs/cell) (mean ± SEM, $n = 3$). The mean IC_{50} values derived from analysis by the LIGAND program of the specific competitive inhibition of binding were 0.9 ± 0.1 nM for PGE_1 and >1 μM for the other prostanoids. The binding of [^{3}H]PGE_2 also was inhibited by the EP_3/EP_1R-selective agonist sulprostone ($IC_{50} = 4.3$ ± 0.2 nM) (mean ± SEM, $n = 3$), the $EP_3/EP_2/EP_4$R-selective agonist M&B 28767 ($IC_{50} = 5.7 \pm 0.4$ nM), which binds preferentially to the EP_3 Rs, and the $EP_2/EP_4/EP_3$R agonist misoprostol ($IC_{50} = 30.9 \pm 1.9$ nM) (Figure 1B). The specific binding of [^{3}H]PGE_2, however, was not inhibited by the EP_1 R-selective antagonist SC-19220 (Figure 1B) or the EP_2 R-selective agonist butaprost at concentrations of up to 10 μM (data not shown). These results demonstrated a predominance of EP_3 and EP_4 Rs and the absence of EP_1 and EP_2 Rs on RNK-16 cells. PGE_2 stimulated an increase in $[cAMP]_i$ in RNK-16 cells, with a concentration dependence that resulted in mean maximal increases at 10 min of (1.8 ± 0.3)-, (4.2 ± 0.8)-, (5.9 ± 0.8)-, and (11.7 ± 2.5)-fold (mean ± SEM, $n = 6-8$, $p > 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.01$, respectively, by a Student's t test) for 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M PGE_2, respectively, when compared with the basal $[cAMP]_i$ of 2.3 ± 0.3 pmol/10^6 cells. The $EP_2/EP_4/EP_3$R agonist misoprostol also stimulated a mean maximal increase in $[cAMP]_i$ of (4.7 ± 1.3)-fold (mean ± SEM, $n = 6$, $p < 0.05$) at a concentration of 10^{-6} M. In contrast, the EP_2 R-selective agonist butaprost or the EP_3 R-selective agonist sulprostone did not significantly stimulate the $[cAMP]_i$ in RNK-16 cells at a concentration of up to 10^{-6} M (data not shown). Of two subtypes of PGE_2 Rs, the EP_2 and EP_4 Rs, that transduce increases in $[cAMP]_i$, the finding that the $[cAMP]_i$ in RNK-16 cells was increased by PGE_2 and misoprostol, but not by butaprost, confirmed the presence of functional EP_4 Rs.

EP_4 R-Dependent PGE_2 Stimulation of Secretion of MMPs by RNK-16 Cells. Gelatin zymographic analyses of the supernatants from RNK-16 cells revealed secretion of the gelatinolytic activities, which were predominantly composed of three components with sizes in the range of 55−59 kDa (Figure 2) that encompasses the molecular mass of MMP-1 and MMP-3 (Birkedal-Hansen et al., 1993). The activities of MMPs were not detected at 4 h but appeared after 24 h when 10^{-7} M PGE_2, but not LTB_4, enhanced MMP activities significantly (Figure 2A). It appeared that the activity of each of the three MMPs was enhanced by PGE_2 to a similar extent, although we could not quantify them separately by densitometry. The amounts of proteins secreted were enhanced by a mean maximal (1.2 ± 0.2)-fold (mean ± SD, $n = 3$) by 10^{-7} M PGE_2, when compared with the control, but this slight increase in the amount of protein used for zymography was not sufficient to account for the significant increases in MMP activities evoked by PGE_2. The total activities of the aggregate of all three MMPs were increased by (2.2 ± 0.3)-fold (mean ± SEM, $n = 5$, $p < 0.05$ by a Student's t test) over control by 10^{-7} M PGE_2, as determined by densitometry (Figure 2A) after being normalized to the same amount of total protein. The activities of MMPs were not further increased after 48 h (data not shown), suggesting that secretion of the MMPs by RNK-16 cells attained a maximal level after 24 h of incubation. Enhancement of the secretory MMP activities at 24 h showed a dependence on PGE_2 concentration, with a significant increase by 10^{-9} M PGE_2 and maximal enhancement by 10^{-8} and 10^{-7} M PGE_2 (Figure 2B). The MMP activities of three components were manifested equally clearly and with the same pattern in gels copolymerized with casein (data not shown) that serves as a favored substrate for MMP-3, when compared with that in gelatin gels that serves as a substrate for all MMPs (Birkedal-Hansen et al., 1993), suggesting that at least one of the three components is MMP-3.

The identities of the three components of the MMP activities were established by Western blot analyses of the secreted MMPs. Two components of the MMP activities (55−59 kDa) were immunochemically identical to MMP-1

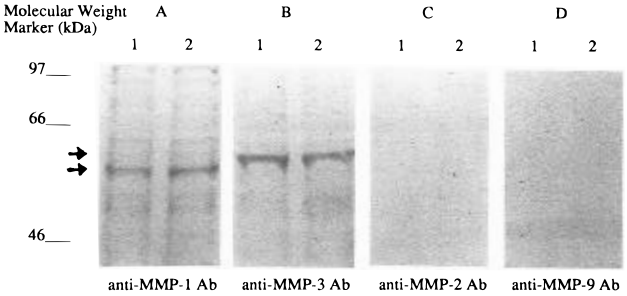

FIGURE 3: Western blot analyses of the MMPs secreted by RNK-16 cells. Each lane was loaded with 5 μg of concentrated supernatant proteins of control (lane 1) and 10^{-7} M PGE_2 treatment (lane 2) at 24 h. Western blot was developed with 1 μg/mL mouse monoclonal IgG antibodies specific for MMP-1 (55−60 kDa, A), MMP-3 (55−60 kDa, B), MMP-2 (72 kDa, C), and MMP-9 (92 kDa, D). The arrows indicate the predominant protein bands that correspond to MMP-3 (upper) and MMP-1 (lower). The result is representative of three experiments.

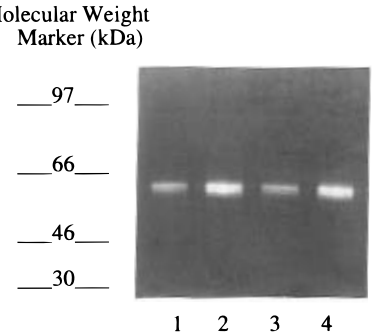

FIGURE 4: Effect of PGE_2 on MMPs secreted by RNK-16 cells is mediated by cAMP. Supernatant protein samples in lanes 1−4 are control and treatment with 10^{-7} M PGE_2, 10^{-6} M sulprostone, and 10^{-4} M dibutyryl-cAMP for 24 h, respectively. Zymographic analyses of the supernatants were performed as described. The result is representative of four experiments.

(Figure 3A) and MMP-3 (Figure 3B), respectively, that correspond in size to the lower and middle bands of the three MMP activities shown by zymography. Of equal importance was the complete absence of the gelatinases MMP-2 (72 kDa) and MMP-9 (92 kDa) (Figure 3C,D), which are prominent MMPs of macrophages and the principal MMPs of T cells (Goetzl et al., 1996). Moreover, the amounts of MMP-1 and MMP-3 immunoreactive proteins did not change upon PGE_2 stimulation when compared with control as determined by densitometry (Figure 3A,B), implying that enhancement of the activities of MMP-1 and MMP-3 by PGE_2 is due to an increase in their specific activities. In addition, 10^{-4} M dibutyryl-cAMP, a permeant cAMP analogue, enhanced the same MMP activities to a similar extent as PGE_2 at 24 h (Figure 4), but concurrent treatment of RNK-16 cells with the EP_3 R subtype-selective agonist sulprostone did not mimic the PGE_2 effect (Figure 4), suggesting that an EP_4 R-dependent mechanism predominated in the cAMP-dependent enhancement of MMP activities by PGE_2. The EP_2/EP_4/EP_3R agonist misoprostol at 10^{-6} M also enhanced the same MMP activities by an aggregate (2.1 ± 0.4)-fold (mean ± SEM, $n = 3$, $p < 0.05$) over control, which further supports the EP_4 R-dependent mechanism of augmentation of MMP activities by PGE_2 in RNK-16 cells.

MMP Dependence of RNK-16 Cell Migration through a Model Basement Membrane. PGE_2-stimulated RNK-16 cell chemokinesis, rather than chemotaxis, as the levels of migration through a micropore filter with (Figure 5, upper

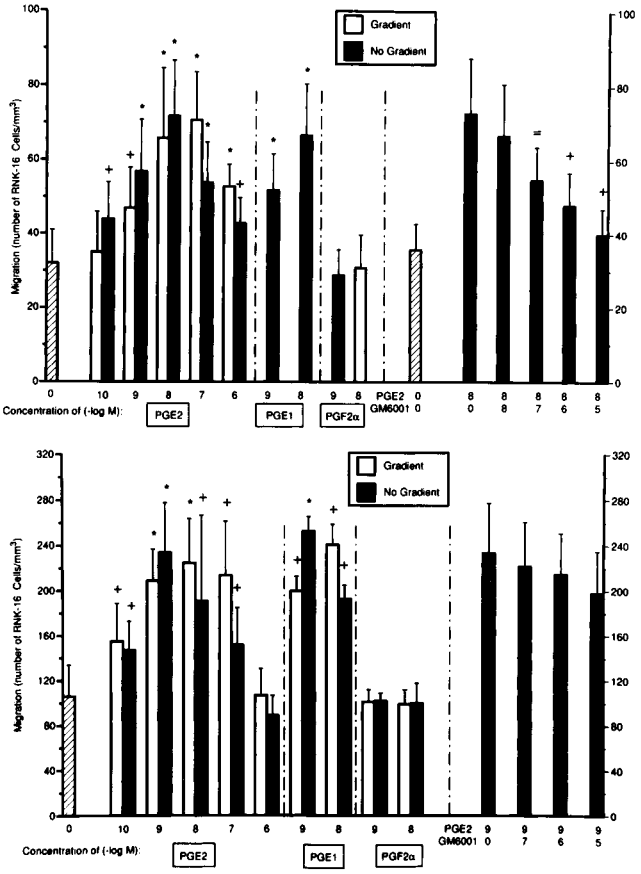

FIGURE 5: MMP dependence of RNK-16 cell migration. Each bar and bracket depicts the mean ± SD of the results of three studies of RNK-16 cell migration with (upper panel) and without (lower panel) Matrigel. Chemotaxis is determined with PGE_2 in only the lower compartment and chemokinesis with PGE_2 in both compartments in the absense of a concentration gradient. The statistical significance of the difference between a stimulated value and the base line response was calculated by a two-sample *t*-test and represented by the following symbols: ∗, $p < 0.01$; +, $p < 0.05$; and =, $p = 0.05$.

panel) or without Matrigel (Figure 5, lower panel) evoked by each concentration of PGE_2 was the same whether PGE_2 was presented in a concentration gradient (open bars of Figure 5) or at the identical concentration on both sides of the filter (closed black bars of Figure 5). Chemokinesis of RNK-16 cells across a micropore filter without Matrigel was stimulated significantly by 10^{-10} to 10^{-7} M PGE_2 and maximally by 10^{-9} M PGE_2 and PGE_1, whereas $PGF_{2\alpha}$ had no effect (Figure 5, lower panel). That the higher concentration of PGE_2 (10^{-6} M) did not stimulate chemokinesis of RNK-16 cells across a micropore filter without Matrigel is typical of the concentration dependence relationship observed for other leukocytes (Snyderman & Goetzl, 1981). Chemokinesis of RNK-16 cells through Matrigel was stimulated significantly by 10^{-10} to 10^{-6} M PGE_2, with similar maximal responses to 10^{-9} and 10^{-8} M PGE_2 and PGE_1, whereas $PGF_{2\alpha}$ had no effect (Figure 5, upper panel). Maximal PGE_2-induced chemokinesis through Matrigel was suppressed significantly by 10^{-7} to 10^{-5} M GM6001 (a hydroxamic acid dipeptide analogue inhibitor of MMPs) (Figure 5, upper panel), with a dependence on GM6001 concentration similar to that observed for inhibition of the activity of MMPs (Leppert et al., 1995a). In contrast, the same concentrations of GM6001 failed to influence significantly the maximal level of PGE_2-induced chemokinesis across a micropore filter

in the absence of Matrigel (Figure 5, lower panel). The control peptide GM2454, which does not inhibit MMPs, had no effect at any concentration on PGE_2-induced chemokinesis of RNK-16 cells through Matrigel. In addition, other protease inhibitors, including 10 μM PMSF, 3 mM leupeptin, and 1 mM DL-thiorphan, alone or in combinations failed to inhibit MMP activities of RNK-16 cells by a mean of more than 19% ($n = 2$) of control, and none of the protease inhibitors had a significant effect on chemokinesis through Matrigel. Furthermore, the PGE_2 effect on chemokinesis of RNK-16 cells was mimicked by the $EP_2/EP_4/EP_3$R agonist misoprostol. At concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M, misoprostol elicited chemokinesis of RNK-16 cells across a micropore filter without Matrigel, with mean maximal stimulation of (1.6 ± 0.2)-, (3.1 ± 0.4)-, (2.2 ± 0.2)-, and (1.3 ± 0.1)-fold, respectively (mean ± SD, $n = 4$, $p < 0.05$, $p < 0.01$, $p < 0.01$, and $p > 0.05$, by a Student's t test), and evoked chemokinesis across a micropore filter with Matrigel with mean maximal stimulation of (1.8 ± 0.5)-, (2.7 ± 0.7)-, (2.3 ± 0.8)-, and (1.4 ± 0.4)-fold (mean ± SD, $n = 4$, $p < 0.05$, $p < 0.01$, $p < 0.01$, and $p > 0.05$, by a Student's t test).

DISCUSSION

We have employed the RNK-16 clonal NK cell line (Seaman et al., 1987; Reynolds et al., 1984) devoid of contaminating cells, as a model for NK cell responses to PGE_2, and characterized for the first time the subtypes of PGE_2 Rs and their effects on MMPs in NK cells. The coexpression by RNK-16 cells of both EP_3 and EP_4 subtypes of PGE_2 R with high affinity and specificity was demonstrated by ligand binding studies and second messenger measurements with PGE_2 R subtype-selective agonists. The EP_3/EP_1 R-selective agonist sulprostone, the $EP_3/EP_2/EP_4$ R-selective agonist M&B 28767, and the $EP_2/EP_4/EP_3$R agonist misoprostol all competed specifically for [^{3}H]PGE_2 binding to intact RNK-16 cells (Figure 1). However, neither the EP_1 R-selective antagonist SC-19220 nor the EP_2 R-selective agonist butaprost inhibited [^{3}H]PGE_2 binding to RNK-16 cells, suggesting a lack of EP_1 and EP_2 Rs on RNK-16 cells. Of the two cAMP-elevating PGE_2 Rs, the EP_2 and EP_4 Rs, expression of functional EP_4 Rs was confirmed by finding that PGE_2 and misoprostol, but not butaprost, evoked increases in $[cAMP]_i$ in RNK-16 cells. Some isoforms of the EP_3 Rs have been reported to stimulate increases in $[cAMP]_i$ (Namba et al., 1993), but this is not the case in RNK-16 cells because the EP_3 R-selective agonist sulprostone did not stimulate increases in $[cAMP]_i$ at a concentration of up to 10^{-6} M (data not shown). Therefore, it is the EP_4 Rs that mediate increases in $[cAMP]_i$ evoked by PGE_2 in RNK-16 cells.

A family of matrix metalloproteinases (MMPs) is the principal physiological system that degrades diverse components of the extracellular matrix, and they are regulated by many factors (Birkedal-Hansen et al., 1993; Woessner, 1994). It has recently been demonstrated that MMP-2 and MMP-9 are expressed by human T cells, and PGE_2 stimulates secretion of MMP-2, -3, and -9 in blood T cells and Tsup-1 cells (Leppert et al., 1995a). Using RNK-16 cells as a model NK cell, we examined the pattern of MMP expression and the ability of PGE_2 to enhance secretion and activities of MMPs in NK cells. Of the three MMP activities secreted by RNK-16 cells, two smaller components were suggested in size and immunochemical status to be MMP-1 and MMP-3 by zymography and Western blot (Figures 2 and 3), whereas the largest component of the MMP activities was not identified immunochemically by any of the probes available, although it also corresponds in size to MMP-1 and MMP-3. In contrast, predominant MMP activities in T cells are MMP-2 and MMP-9 (Goetzl et al., 1996; Leppert et al., 1995a), although MMP-3 (stromelysin-2) transcripts have also recently been found in T cells after exposure to phorbol myristate acetate and calcium ionophore (Conca & Willmroth, 1994). Thus, RNK-16 cells showed a unique profile of secretory MMP activities that differs completely from that of T cells. RNK-16 cells constitutively secrete MMP-1 and -3, and the activities of both secreted MMPs, as assessed by zymography, were enhanced by PGE_2 after 24 h of incubation in a dose-dependent manner; on the other hand, parallel Western blot analyses of the secretion of MMP proteins by RNK-16 cells showed that the amounts of secretory MMPs did not change significantly. PGE_2 exerts its effects on MMPs in RNK-16 cells by stimulating enzymatic specific activities rather than increasing the amount of secretory protein. The biologic activation of MMPs is still not fully understood, although evidence suggests that activation is mainly a result of conformational changes in MMPs (Birkedal-Hansen et al., 1993). PGE_2 might induce conformational changes in MMP-1 and -3, but these have not been examined. An effect similar to that of PGE_2 on activities of MMPs in RNK-16 cells was also observed with the $EP_2/EP_4/EP_3$R agonist misoprostol and PGE_1, but not with the other synthetic prostanoids $PGF_{2\alpha}$, PGD_2, or PGI_2, the EP_2 R-selective agonist butaprost (data not shown), the EP_3 R-selective agonists M&B 28767 and sulprostone (Figure 4), or LTB_4 (Figure 2), suggesting that the effect of PGE_2 on MMP activities in RNK-16 cells is specific.

The diverse cellular effects of PGE_2 are mediated by PGE_2 Rs of four subtypes, the EP_1, EP_2, EP_3, and EP_4 Rs (Coleman et al., 1994). The coexpression of both EP_3 Rs and EP_4 Rs in RNK-16 cells permitted studies of distinctive roles of subtypes of PGE_2 Rs on the MMPs in NK cells. The PGE_2 effect on secreted MMPs in RNK-16 cells was mimicked by the $EP_2/EP_4/EP_3$R agonist misoprostol and dibutyryl-cAMP, but not by the EP_3 R-selective agonist sulprostone (Figure 4). Thus, the principal effect is mediated by the cAMP-elevating EP_4 Rs expressed by RNK-16 cells. Increases in $[cAMP]_i$ also were reported to be the mechanism by which PGE_2 inhibited the lytic capabilities of NK cells (Brunda et al., 1980; Goto et al., 1983; Roder & Klein, 1979; Vaillier et al., 1992, 1994).

The cell surface expression and secretion of MMPs are important for the migration in immune cells (Goetzl et al., 1995). In this study, we examined the ability of PGE_2 to stimulate migration in RNK-16 cells through Matrigel with a dependence on endogenous MMPs and demonstrated that PGE_2 stimulated RNK-16 cell chemokinesis but not chemotaxis in a dose-dependent manner. PGE_2-evoked chemokinesis of RNK-16 cells through Matrigel was suppressed significantly by an inhibitor of MMPs, GM6001 (Figure 5, upper panel), with a dependence on GM6001 concentration similar to that observed for inhibition of the activity of MMPs (Leppert et al., 1995b). However, the inhibitors of serine proteases of tryptic, chymotryptic, and other types, as well as proteases of other classes in granules of NK cells, had no significant effect on chemokinesis through Matrigel. The

marked parallel suppression by GM6001 of MMP activities and chemokinesis of RNK-16 cells through Matrigel supports a major role for MMPs in RNK cell migration. Secretion of MMP-1 and -3, thus, appears to be required for migration of RNK-16 cells through the model basement membrane.

In summary, we have shown for the first time that a rat model NK cell, RNK-16, coexpresses the EP_3 and EP_4 subtypes of PGE_2 Rs and that PGE_2 activates MMP-1 and MMP-3 of RNK-16 cells through an EP_4 R-dependent mechanism, which also mediates the chemokinetic effect of PGE_2 on transmigration of RNK-16 cells through a model vascular basement membrane.

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