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The Influence of Mast Cell Mediators on Migration of SW756 Cervical Carcinoma Cells

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Abstract. The role of mast cell mediators on cervical cancer cell migration was assessed using an in vitro assay of scratch wound healing onto monolayers of HPV18-positive cervical carcinoma cells (SW756). Migration of SW756 cells was accelerated by co-culture with the mast cell line LAD2. This effect was inhibited by the H1R antagonist pyrilamine and the cannabinoid agonists 2-arachidonylglycerol (2AG) and Win 55,212-2. Therefore, the specific effects of histamine and cannabinoids on SW756 migration and LAD2 activation were analyzed. Histamine added to the in vitro assay of scratch wound healing either increased or inhibited SW756 migration rate by acting either on H1R or H4R, respectively. Cannabinoids acted on CB1 receptors to inhibit SW756 migration. Supernatants from SW756 cells stimulated LAD2 cell degranulation, which in turn was inhibited by cannabinoids acting via CB2 receptors. RT-PCR showed that SW756 expressed mRNA for CB1, CB2, H1R, H2R, and H4R. On the other hand, LAD2 expressed mRNA for all four HRs and CB2. The results suggest that mast cells could be contributing to cervical cancer cell invasion and spreading by the release of histamine and cannabinoids. Therefore, therapeutic modulation of specific mast cell mediators may be beneficial for cervical cancer treatment.

Keywords: mast cell, cervical cancer, histamine, cannabinoid, cell migration

Introduction

Cell-mediated immune responses are essential for the control of human papilloma virus (HPV)-related neoplasias such as cervical cancer (1). Morphologic studies have revealed that mast cells accumulate at the tumor-host junction in several types of tumors including the malignant lesions of the uterine cervix. High numbers of active, degranulated mast cells have been described in HPV infections and cervical intraepithelial neoplasias (2 – 4). Hence, a functional relationship between mast cells and tumor cells has been proposed (3), where mast cells are involved in stimulating tumor growth and progression by enhancing angiogenesis, immunosuppression, stromal remodeling, mitogenesis, and metastasis (5). In addition, it has been suggested that mast cells participate in a bidirectional cross-talk with the tumor cells, which recruit mast cells into the tumor and stimulate their proliferation and degranulation (6).

Mast cell activation leads to the release of inflammatory mediators, including histamine. Increased histamine levels have been described in colorectal, breast, and skin cancers (7 – 9), where they have been associated with tumor growth and progression (10). Histamine receptors have been reported in different cell lines and tissues derived from experimental and human neoplasias. Based on their pharmacological and signal transduction mechanisms, four receptor types, H1R through H4R, have been described, all of which belong to the G-protein–coupled receptor family (11, 12). H1R is preferentially coupled to $G\alpha_{q/11}$ proteins, leading to increased Ca^{2+} mobilization and activation of PKC via the phospholipase C / inositol-triphosphate / diacylglycerol pathway (11). In addition, H1R has the potential to bind to $G\alpha_s$ that stimulates adenylyl cyclase to generate cAMP, the substrate of PKA activation (13). PKC and

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PKA can phosphorylate various proteins playing a fundamental role in the regulation of cell shape and migration (14). It has been found that H1R stimulates proliferation of breast carcinoma, melanoma, and astrocytoma tumor cells (15 – 17). H2R is coupled to $G\alpha_s$, leading to adenylyl cyclase system activation and increased cAMP production (11). Regarding the effect of the activation of H2R on tumor growth and proliferation the results are controversial. Indirect experiments, by the use of H2R antagonists, have suggested that this receptor could stimulate cancer development. In fact, cimetidine (an H2R antagonist) has shown to reduce growth and proliferation of human glioma, melanoma, and colon carcinoma cells (18 – 20) and also to have a beneficial effect as an adjunct to surgical excision in patients with a variety of neoplasias (21). Nevertheless, very recent results have also shown that activation of H2R exerts an antiproliferative effect on human pancreatic cancer cells via activation of Bcl-2 family proteins (22). H3R and H4R have relatively high homology and are coupled to $G\alpha_o$ and $G\alpha_i$ proteins, leading to adenylyl cyclase inhibition and cAMP reduction, therefore reducing ability of the cell to activate protein kinases (11, 23). H3R participates in the protection of gastric mucosa by a direct action on gastric epithelial cells (24, 25) and in regulating neurotransmitter release (11), but its role in carcinogenesis has not yet been determined. H4R is involved in inflammatory responses (26) and is present in epithelial cells of normal mammary glands and breast neoplasias, where it may play a protective role against tumor progression (27).

Mast cells are also involved in the regulation of endocannabinoid synthesis and uptake (28, 29). Synthetic cannabinoids such as Win 55,212-2 and endocannabinoids like 2-arachidonoylglycerol (2AG) exert antiproliferative actions over a wide spectrum of tumor cells and are described as potential anticancer agents (30). Nevertheless, recent findings also suggest a possible proliferative effect of cannabinoids at lower doses (29, 31). Cannabinoids are known to mainly activate G-protein–coupled receptors, through $G\alpha_{i/o}$ proteins, leading to adenylyl cyclase inhibition (32). Their effect on tumor cell migration is controversial: they modulate cell migration through the cannabinoid receptors CB1 and CB2, with stimulatory or inhibitory effects depending on the cell type. The endocannabinoid anandamide inhibits migration of a human carcinoma cell line (SW480) through CB1 receptors (33), and 2AG increases HL-60 and murine myeloid leukemia cell migration through CB2-dependent mechanisms (34, 35). On the other hand, cannabidiol inhibits human glioma cell migration through a CB-independent mechanism (36).

Tumor invasion and metastasis are the most common causes of death in cancer patients. These processes include changes both at the tumor stroma, such as remodeling and degradation of extracellular matrix components, and in tumor cells, which express a migratory phenotype that enables them to loosen cell-cell adhesion and increase motility. Different experimental models have been designed to analyze the mechanisms involved in cell motility and invasion (14, 37). The in vitro wound healing assay has been shown to be a useful model for analyzing the non-proteolytic process. In this model, individual cells use a mesenchymal-type migration towards the wound, at a rate that can be monitored through image analysis during the experiment (38). Being the wound has a constant stimulus for motility and invasion, migration can be stimulated or inhibited by acting on the tumor cell receptors that affect mostly adhesion molecules or cytoskeletal components (39 – 41). Numerous studies have shown the crucial role of mast cells in tumor development by altering extracellular matrix remodeling (42), but no studies have been performed on the effect of mast cells on tumor cells migration. Therefore, the aim of this study was to determine in vitro whether the mature mast cell line LAD-2 could regulate malignancy by affecting the migratory potential of the cervical carcinoma cell line SW756 (an HPV18-positive cervical carcinoma cell line) (43). In addition, histamine and cannabinoid receptor function and expression were assessed in SW756 and LAD2 cell lines by pharmacological assays and RT-PCR, respectively.

Materials and Methods

Media and reagents

L-15 Leivobitz, penicillin, streptomycin, amphotericin B, glutamine, StemPro-34 complete medium, fetal bovine serum (FBS), TRIzol, trypan blue, and trypsin/EDTA were purchased from Invitrogen (Gibco, Rockville, MD, USA). ^{3}H-thymidine was obtained from New England Nuclear Corp (Boston, MA, USA). 2-Arachidonoylglycerol (2AG), phenylmethylsulfonylfluoride (PMSF), *R*-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethylmethanone (Win 55,212-2), histamine hydrochloride, pyrilamine hydrochloride, and ranitidine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Thioperamide and *N*-(piperidin-1-y)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidem (AM251) were obtained from Tocris Cookson Ltd. (Bristol, UK). Enzyme-linked immunosorbent assay kit (ELISA) for histamine was obtained from Biosource Europe S.A. (Nivelles,

Belgium). RNeasy MinElute Spin columns were bought from Qiagen, Inc. (Valencia, CA, USA). *N*-[(1*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide (SR144528) was a gift from Sanofi Recherche (Montpellier, France). Stem cell factor (SCF) was kindly donated from Amgen, Inc. (Thousand Oaks, CA, USA). The HPV-18 positive human cervical carcinoma SW756 cell line was purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA) and the human mast cell line LAD2 cells was kindly provided by Professor Dr. D.D. Metcalfe, from the Center for Cancer Research, National Institutes of Health (Bethesda, MD, USA).

PMSF, AM251, SR144528, and thioperamide were first dissolved in dimethyl sulfoxide (DMSO). Final concentrations of DMSO in all experimental samples was less than 0.1%. 2AG was first dissolved in acetonitrile, with a final concentration in all experimental samples of less than 0.01%. Compounds were freshly dissolved for each experiment from concentrated stocks stored at −20°C for less than 3 months. 2AG was administered together with PMSF at 100 μM for hydrolase inhibition. LAD2 is a recently described mast cell line developed from the bone marrow of a patient with mast cell leukemia. The cells were maintained in culture with StemPro-34 complete medium supplemented with glutamine (2 mM), penicillin (100 IU/mL), streptomycin (50 μg/mL), and SCF (100 ng/mL) as described previously (44).

Wound healing assay

Culture conditions for the HPV-18–positive human cervical cancer cell line, SW756, were optimized to ensure a homogeneous and viable cell monolayer prior to wounding. A starved culture was used in order to ensure reproducible measurements of the wound edge movement into the empty wound space with no detectable cell proliferation and no cell viability loss over the experimental time period (38).

SW756 cells were grown according to ATCC instructions at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2 in L-15 Leivobitz, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (complete medium: L-15). For migration analysis, cells were detached by trypsinization, resuspended in the same medium and plated in 24-well plates at 250,000 cells/well in a final volume of 0.5 mL. After the culture became confluent, cells were starved by omitting FBS (serum-free medium: L-15 SFM) for 18 h, and then an artificial wound was created onto the monolayer with a sterile plastic 10-μL micropipette tip to generate one homogeneous wound along each well. After wounding, tissue culture media was removed, and SW756 cells were washed at least twice to eliminate detached cells. Experiments were performed by incubating the SW756 cells with LAD2-mast cells at different densities, from 200:1 to 2:1 or with L-15 SFM alone (control). In another set of experiments, L-15 SFM was supplemented with the compounds histamine (1 – 300 μM), pyrilamine (10 μM), ranitidine (10 μM), 2AG (1 – 100 nM) + PMSF (100 μM), SR144528 (500 nM), AM251 (100 nM), Win 55,212-2 (1.0 – 100 μM), or thioperamide (10 μM) to evaluate the effect of each compound on cell migration post-wounding. For every experiment, each plate had its own internal control. Antagonists were added 20 min before the agonists. Wound edge movement was monitored across the wound at the time of wounding and 24 h after with a phase-contrast microscope (Nikon Diaphot 300; Nikon, Tokyo) connected to a digital camera (CoolSNAP-Pro; Media Cybernetics, Bethesda, MD, USA). Microphotographs were captured and analyzed by videomicroscopy and image analysis (described in detail in the next section). Viability was evaluated at the end of each experiment. Within one experiment, 3 – 4 duplicate determinations were made for each incubation condition.

Videomicroscopy and image analysis

Images were captured with a light microscope (magnification of 4×) with phase-contrast illumination (Nikon Diaphot 300) coupled to a digital camera (CoolSNAP-Pro) and interfaced with a host computer and image processing and recording systems (Image-Pro Express software, Media Cybernetics). Before measuring, distance calibration was defined using a slide with a micrometric scale for microscopy (Rechert, Vienna, Austria). Distances between the edges were measured by using the image analysis software at 50-μm intervals all along the wound. A minimum of 36 readings were obtained for each well. Quantitative cell migration assays were performed in duplicate wells and each experiment was repeated at least three times. This system provided sufficient resolution to determine the extent of migration and the morphology of the cells at the wound margin.

Cell proliferation assay

Cells were plated in 96-well plates in triplicates at a density of 75,000 cells/well in 150 μL of tissue culture medium. After reaching confluence, cells were incubated in L-15 SFM for 18 h. Then cells were treated with ^{3}H-thymidine (0.45 μCi) and supplemented with the compounds previously described. After 24 h of incubation, cells were detached with 2.5 g/L trypsin /0.38 g/L EDTA and washed to analyze the incorporation of ^{3}H

into the cells, which became an index of proliferation.

Trypan blue assay

For evaluation of cell viability, SW756 and LAD2 cells were treated with 0.4% trypan blue and immediately evaluated under the microscope to assess dye exclusion.

Immunohistochemical detection of mast cells

For immunohistochemical detection of mast cells in co-culture with SW756 cells for 24 h, cells were washed and fixed with 10% neutral formaldehyde for 30 min. After permeabilizing with 0.1% Triton X-100, cells were processed for immunohistochemical detection of tryptase-positive mast cells, as previously described (45).

LAD2 mast cell stimulation and β-hexosaminidase and histamine assays

β-Hexosaminidase release was determined after incubating LAD2 mast cells for 24 h with L-15 SFM or the supernatant of SW756 cells incubated for 18 h in L-15 SFM. As a positive control, LAD2 mast cells were incubated for 10 min in L-15 SFM plus compound 48/80 (3 μg/mL). LAD2 mast cells (10,000) were seeded in 96-well microtiter plates and subsequently incubated as described. The 50-μL aliquots of culture supernatants of LAD2 cells lysed with 0.1% Triton X-100 were treated to determine *β*-hexosaminidase by adding 50 μL 2 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide in 0.2 M citrate-buffer (pH 4.5) as substrate. After a 2-h incubation at 37°C, the reaction was stopped with 150 μL of 0.4 M glycine and absorbance was determined at 405 nm. Percentage of release was determined as already described (46). The levels of histamine released by LAD2 cells or present in the supernatants of SW756 cell cultures were determined with an Enzyme Immunoassay Kit (Histamine EIA, Biosource) according to the manufacturer's instructions.

RT-PCR analysis

Total RNA was isolated from SW756 and LAD2 cells using TRIzol (Life Technologies, Rockville, MD, USA) and following the manufacturer's instructions. RNA concentration was determined spectrophotometrically by UV absorbance. RNA samples (10 μg) were applied to RNeasy MinElute Spin columns (Qiagen, Valencia, CA, USA) followed by elution with RNAse-free water, according to the manufacturer's specifications. All total RNA samples were reverse-transcribed to cDNA as previously described (38). Expressions of histamine receptor (H1R, H2R, H3R, and H4R) and cannabinoid receptor (CB1 and CB2) mRNAs were determined by PCR using the primer sequences shown in Table 1. PCR conditions used 45 PCR cycles that were run at 94°C (60 s), 55°C (45 s), and 72°C (60 s) for H1R, H2R, H3R, H4R, CB1, and CB2 and 35 cycles that were run at 94°C (60 s), 60°C (45 s), and 72°C (60 s) for GAPDH. PCR products were electrophoresed in a 1.5% agarose gel, visualized by means of ethidium bromide staining under UV, and photographed.

Statistical analyses

For each experiment, the effect of the different compounds on SW756 cells migration rate was expressed as wound closure rate (μm/h), considered as

Table 1. Primer sequences

Product (size)	Reference	Primer sequence 5'-3' Forward Primer sequence 5'-3' Reverse
GAPDH (301 bp)	58	CGGAGTCAACGGATTTGGTCGTAT AGCCTTCTCCATGGTTGGTGAAGAC
H1R (492 bp)	59	TAAGCTGAGGCCAGAGAACC TACTGTCTTGAATGCGAGCG
H2R (498 bp)	59	GCACAGCCTCCATTCTTAACC CCTCATTGATGGCATCATCC
H3R (779 bp)	59	TCCTCTGCCTTCAACATCG ATCATCAGCAGCGTGTATGG
H4R (522 bp)	59	CATCCCTCACACGCTGTTCG GCAGGAACTTCTGTCGATGC
CB1 (398 bp)	60	CCAGTGTTCACAGGGCCGCAG GGGTTCTTAGACTTCCAATTGTGTAGGCC
CB2 (676 bp)	61	GACCTTCACAGCCTCTGTGGGTA GATTTTCCCATCAGCCTCTGTCT

half the distance measured between wound sides. Statistical analyses were performed using the JMP-IN 4.0.4 statistical program (SAS Institute, Inc., Cary, NC, USA). Differences between groups were determined with the *t*-test or ANOVA and Tukey-Kramer tests for comparison among multiple groups. Differences were considered statistically significant when $P<0.05$. The results were expressed as the mean ± S.E.M. or mean ± S.D. as specified in the results section.

Results

Human HPV-18–positive SW756 cervical carcinoma cells formed an adherent monolayer that stopped proliferating by exclusion of FBS from the culture media, as measured by ^{3}H-thymidine incorporation (data not shown). One of the main activities detected in the in vitro culture of wounded SW756 cervical carcinoma cells was migration across the wound bed, with similar size and morphology as cells growing before confluence. In addition, the cells exhibited an elongated morphology during migration, with the longitudinal axis polarized toward the wound, a sign of normal migration. As shown in Fig. 1, in each experiment a uniform edge of migrating cells was observed at the wound margin, with a basal migration rate of 2.14 ± 0.57 μm/h (n = 17).

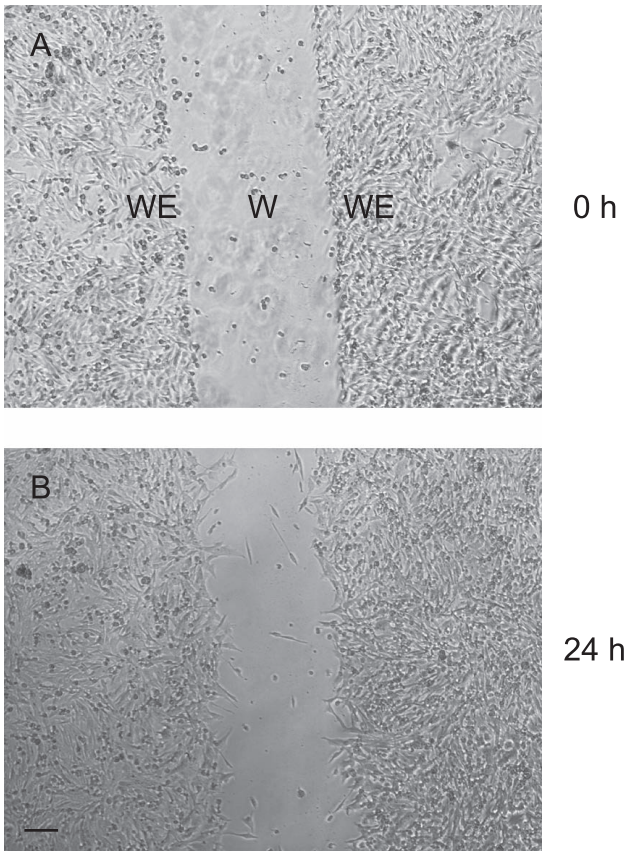

Fig. 1. Representative phase-contrast images of the in vitro wound healing assay. SW756 cervical carcinoma cells were grown to confluence into a monolayer in 24-well plates and then incubated in L-15 SFM for 18 h to inhibit proliferation without affecting migration. A linear scratch wound, performed along the culture well, created a space that allowed SW756 cells to migrate into. The distance between the edges was monitored by digital photographs taken with a phase-contrast microscope across the wound at the moment of wounding and at 24 h post-wounding. Images were analyzed and migration rate was obtained as described in Methods. 0 h and 24 h: wound at time 0 and at 24 h after wounding, respectively. W = wound space, WE = wound edge. Scale bar, 100 μm.

Effect of the mast cell line LAD2 on migration rate of SW756 cells

To assess the effects of LAD2 cells on SW756 cells migration, LAD2 cells at different densities (ranging from 3×10^3 to 3×10^5 cells) were added to wounded SW756 cell monolayers. Immunohistochemical detection of tryptase-positive LAD2 cells in the co-culture media showed no adhesion of mast cells to SW756 cells (data not shown). To obtain increased SW756 cell migration, the optimum proportion of LAD2:SW756 ranged from 1:60 to 1:6 (Fig. 2) ($P<0.01$, ANOVA and Tukey-Kramer tests).

As shown in Table 2, the stimulatory effect of 1×10^4 LAD2 cells on SW756 cell migration rate was inhibited by the addition of pyrilamine (10 μM), 2AG (100 nM, in the presence of 100 μM PMSF), and Win 55,212-2 (30 μM) ($P<0.05$, *t*-test). On the other hand, no significant inhibition was observed after addition of thioperamide (10 μM), AM251 (100 nM), or SR144528 (500 nM).

Effect of histamine on SW756 cell migration rate

Since pyrilamine inhibited SW756 migration rate evoked by mast cells, the effect of histamine and their antagonists on migration rate of SW756 cell cultures was analyzed. Results are shown in Fig. 3. Although histamine, at a concentration of 10 – 300 μM, did not increase SW756 cell migration rate as compared to media alone, the addition of pyrilamine (10 μM), an H1R antagonist, in the presence of histamine, significantly inhibited migration rate of SW756 cells ($P<0.01$, ANOVA and Tukey-Kramer tests). Ranitidine, an H2R antagonist, at a concentration of 10 μM did not change the effect of histamine on SW756 cells migration, whereas the H3R/H4R antagonist thioperamide, at a concentration of 10 μM, significantly increased SW756 cells migration rate in the presence of histamine ($P<0.01$, ANOVA and Tukey-Kramer tests). None of the antagonists had any effect on the migration rate of SW756 cells when added alone, without histamine (data not shown).

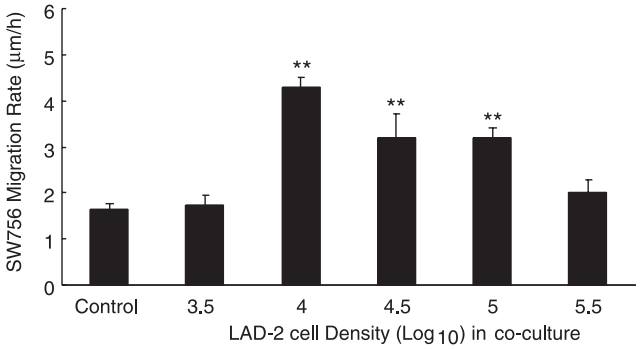

Fig. 2. Effect of LAD2 mast cells on migration rate of SW756 cells. SW756 cells were grown to confluence, starved in L-15 SFM for 18 h, wounded, and co-cultured with increasing densities of LAD2 mast cells for 24 h, as described in Methods. The distance between the edges was monitored in digital photographs taken with a phase-contrast microscope across the wound at the time of wounding and 24-h post-wounding. Images were analyzed and the rate of migration (μm/h) was measured as described in Methods. Results are expressed as the mean ± S.E.M. of three independent experiments performed in duplicate. **P<0.01 (ANOVA and Tukey Kramer tests), as compared to SW756 cells incubated without LAD2 cells.

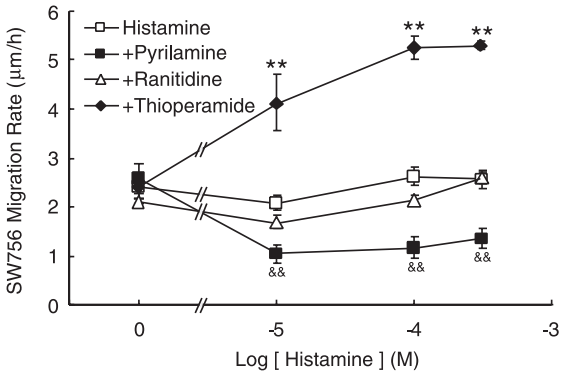

Fig. 3. Effect of histamine on SW756 cells migration rate. The in vitro assay of scratch wound healing onto monolayers of SW756 cervical carcinoma cells was performed as described in methods. Histamine (10 – 300 μM) added to the culture media had no effect on SW756 carcinoma cell migration rate. Histamine antagonists, pyrilamine (10 μM), ranitidine (10 μM), and thioperamide (10 μM) were added to the culture 20 min before histamine. Results are the mean ± S.E.M. of at least three independent experiments performed in duplicate. **P<0.01 (ANOVA and Tukey Kramer tests) for histamine + thioperamide as compared to histamine alone, i.e., SW756 cells incubated in the presence of histamine at corresponding concentrations. $^{\&\&}P$<0.01 (ANOVA and Tukey Kramer tests) for histamine + pyrilamine as compared to histamine

Table 2. Effect of histamine antagonists and cannabinoid agonists and antagonists on LAD2 mast cell–evoked SW756 cell migration

	Migration rate μm/h (mean ± S.D.)
Mast cells alone	4.31 ± 0.64
+ Pyrilamine (10 μM)	2.59 ± 0.43*
+ Thioperamide (10 μM)	4.26 ± 0.96
+ 2-AG (100 nM) + PMSF (100 μM)	2.97 ± 0.28*
+ Win 55,212-2 (30 μM)	1.67 ± 0.45*
+ AM251 (100 nM)	4.80 ± 0.75
+ SR144581 (500 nM)	4.56 ± 0.48

*P<0.05 (t-test), as compared to the effect of mast cells alone, when 10,000 mast cells were co-cultured with 600,000 SW756 cells (ratio SW756/LAD2 mast cells = 60/1). Results are the mean of 3 independent experiments done in duplicate.

Effect of cannabinoids on SW756 cell migration rate

As shown in Fig. 4A, addition of the endocannabinoid 2AG inhibited SW756 cell migration rate in a concentration-dependent manner (concentration range from 10 to 100 nM in the presence of 100 μM PMSF) (P<0.01, ANOVA and Tukey-Kramer tests). Addition of AM251 (100 nM), a CB1-selective antagonist, reverted the inhibitory action of 2AG (P<0.01, ANOVA and Tukey-Kramer tests), while the CB2 antagonist SR144528 (500 nM) had no effect.

As shown in Fig. 4B, Win 55,212-2, a synthetic cannabinoid, at concentrations ranging from 10 to 30 μM, had a similar inhibitory effect as 2AG as compared to control media (P<0.01, ANOVA and Tukey-Kramer tests). Its inhibitory action on SW756 cell migration rate was also reverted by AM251 (100 nM) (P<0.05, ANOVA and Tukey-Kramer tests), but no effect was observed after incubation with SR144528 (500 nM). Neither PMSF (100 μM) nor DMSO and acetonitrile, at the concentration used as vehicles, modified the control migration rate of SW756 cells as measured in L-15 SFM (data not shown).

Mast cell activation by cervical cancer cell supernatants

In order to assess if SW756 cell supernatant was able to stimulate mast cell degranulation, LAD2 cells were incubated for 24 h with the supernatants from SW756 cells treated for 18 h with L-15 SFM. As shown in Table 3, basal release of β-hexosaminidase, a mast cell granule marker (46), was 14.5 ± 1.9%, as determined by incubation of LAD2 cells with L-15 SFM alone for 24 h. Compound 48/80 (3 μg/mL) and SW756 cell supernatant evoked a significant increase in the mean percentage release of β-hexosaminidase of 37.3 ± 4.5 and 26.3 ± 8.2, respectively (P<0.05, as compared to the control, t-test).

As also presented in Table 3, only the addition of the cannabinoid agonist Win 55,212-2 (30 μM) reverted the stimulatory effect of SW756 cells supernatant on LAD2 cell degranulation (P<0.05, t-test). The inhibitory effect of Win 55,212-2 on LAD2 degranulation in response to SW756 cell supernatant was abolished by the CB2 antagonist SR144528 (500 nM) (P<0.005,

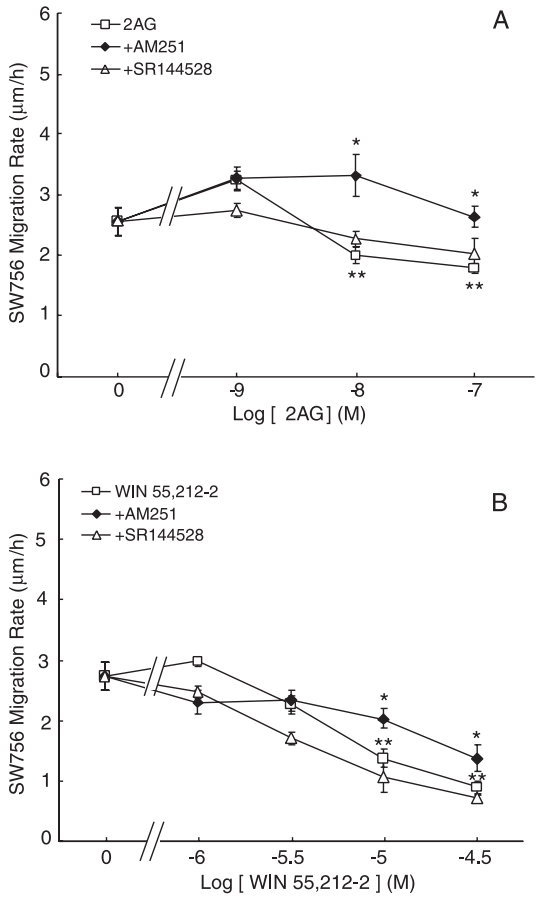

Fig. 4. Effect of 2AG and Win 55,212-2 on SW756 cell migration rate. The in vitro assay of scratch wound healing onto monolayers of SW756 cervical carcinoma cells was already described in methods. A) Effect of the addition of 2AG (10 – 100 nM) in the presence of the hydrolase inhibitor PMSF (100 μM) alone or in combination with the CB1 antagonist AM251 (100 nM) or the CB2 antagonist SR144528 (500 nM) on SW756 cell migration rate. B) Effect of the addition of Win 55,212-2 (1.0 – 30 μM) alone or in combination with AM251 (100 nM) or SR144528 (500 nM) on SW756 migration rate. Results are the mean ± S.E.M. of at least three independent experiments performed in duplicate. ***P*<0.01 (ANOVA and Tukey Kramer tests) for 2AG or WIN 55,212-2 as compared to SW756 cells incubated in the absence of 2AG or WIN 55,212-2. **P*<0.05 (ANOVA and Tukey Kramer tests) for AM251 as compared to 2AG or WIN 55,212-2 alone.

t-test), but not by the CB1 antagonist AM251 (100 nM). Histamine alone and histamine antagonists had no effect on LAD2 β-hexosaminidase release evoked by SW756 cell supernatants.

Determination of histamine content in unstimulated LAD2 cells incubated in L-15 SFM by RIA showed that mast cells contained an average of 8.4 ± 2.3 pg per cell (n = 3). Stimulation of LAD2 cells with 3 μg/mL Compound 48/80 and supernatants from SW756 cells incubated with L-15 SFM evoked an increase of 78.1 ± 14.4% and 43.6 ± 9.8%, resulting a final histamine concentration of 0.70 ± 0.12 and 0.42 ± 0.04 μM, respectively (n = 3). Histamine concentration in the coculture of LAD2 and SW756 cells was 0.36 ± 0.08 M (n = 3) and that in the SW756 cell supernatant was 1.2 ± 0.56 nM (n = 3).

Table 3. Effect of histamine, histamine antagonists, and cannabinoid antagonists on mast cell activation evoked by the 18-h L-15 SFM supernatant of SW756 cells

	% β-Hexosaminidase release[1] (mean ± S.D.)
Control, L-15 SFM	14.5 ± 1.9
18-h L-15 SFM supernatant of SW756 cells	26.3 ± 8.2[#]
+ Histamine (10 μM)	19.9 ± 5.5
+ Pyrilamine (10 μM)	21.2 ± 3.7
+ Ranitidine (10 μM)	20.0 ± 2.1
+ Thioperamide (10 μM)	19.3 ± 1.1
+ Win 55.212-2 (30 μM)	13.5 ± 3.1*
+ Win 55,212-2 (30 μM) + SR144528 (500 nM)	18.4 ± 5.8
+ Win 55,212-2 (30 μM) + AM251 (100 nM)	14.0 ± 3.1*
+ SR144528 (500 nM)	17.9 ± 3.4
+ AM251 (100 nM)	18.8 ± 2.3
L-15 SFM + Compound 48/80 (3 μg/mL)	37.3 ± 4.5[#]

[1]% release of β-hexosaminidase (β-hex) was evaluated as [β-hex in supernatant] × 100 / [β-hex in supernatant] + [β-hex in pellet]. [#]*P*<0.05 (*t*-test), as compared to control, LAD2 mast cells incubated for 24 h in L-15 SFM. **P*<0.05 (*t*-test), as compared to the effect of L-15 SFM supernatant of SW756 cells on β-hexosaminidase release from LAD2 mast cells. Results are the mean ± S.D. of 3 independent experiments performed in duplicate.

Histamine and cannabinoid receptor mRNA expression in SW756 and LAD2 cells

As shown in Fig. 5A, SW756 cells expressed H1R and H2R mRNAs after incubation with L-15 or L-15 SFM. H4R mRNA was only detected in SW756 cells incubated with L-15 SFM, while H3R mRNA was not detected. Similar to H1R and H2R mRNAs, SW756 cells expressed mRNAs for both CB1 and CB2 regardless of the presence or absence of serum (Fig. 5B). LAD2 cells expressed mRNAs for the four histamine receptors (Fig. 5A) and the CB2 receptor. CB1-receptor mRNA was not detected in LAD2 cells (Fig. 5B).

Discussion

Mast cells represent an important component of the lymphoreticular infiltrate of cervical cancer. Their role on migration rate of a cervical carcinoma cell line SW756 (HPV18-positive) was analyzed pharmacologically through the action of two mast cell-related compounds, histamine and cannabinoids. The experi-

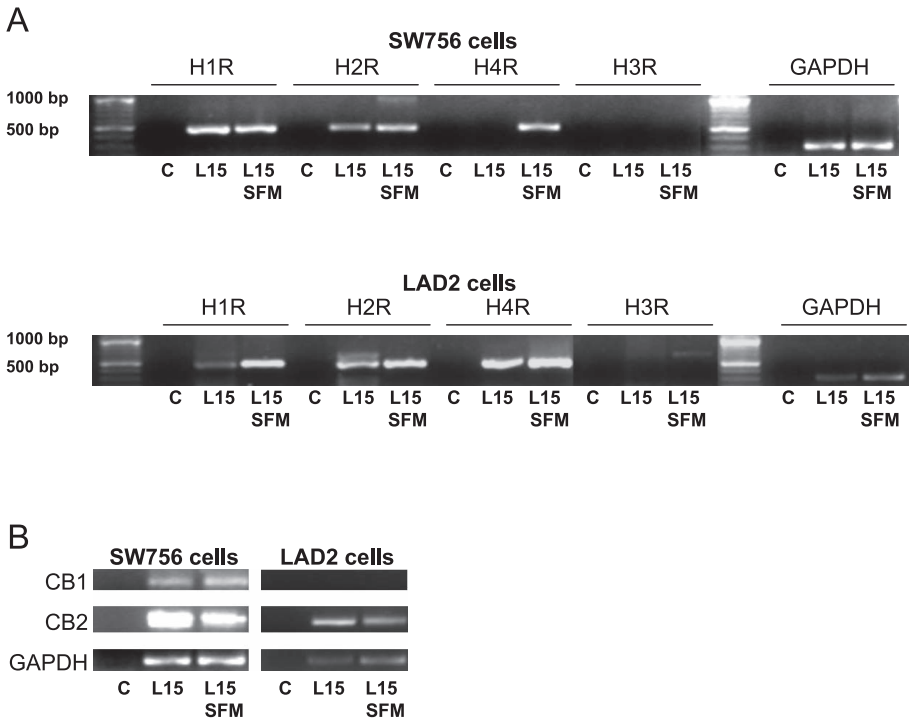

Fig. 5. SW756 cells express mRNA for H1R, H2R, H4R, CB1, and CB2 receptors; and LAD2 cells express mRNA for H1R, H2R, H3R, H4R, and CB2 receptors. Total RNA was isolated from both cultures maintained in complete media (L-15) or serum-free media for 18 h (L-15 SFM) were subjected to reverse transcription and PCR as described in Methods. A) Expression of H1R (492), H2R (498), H3R (779), and H4R (522) specific mRNAs in SW756 and LAD2 cells. B) Expression of CB1 (398) and CB2 (676) specific mRNAs in SW756 and LAD2 cells. C: negative control.

mental model consisted of an in vitro wound healing assay performed on a monolayer epithelial cell culture (38). This model has been proven to be useful in analyzing the mechanisms involved in cell mobility and tissue architecture that may contribute to a malignant phenotype of tumor cells (39 – 41).

To perform coculture studies between mast cells and SW756 cells, the cognate mast cell line LAD2 was used. LAD2 cells were obtained from a mature mast cell line isolated from the bone marrow of a patient with mast cell leukemia (44). Co-incubation of SW756 cells with LAD2 cells increased their migration rate in a concentration-dependent manner. The highest stimulatory effect was observed when the ratio of LAD2:SW756 cells was 1:60, but ratios of 1:10 and 1:6 were also significantly effective. These ratios were similar to the ones already described in studies showing a functional interaction between airway smooth muscle cells and LAD2 cells (47). In addition, a bidirectional interaction between tumor SW756 cells and LAD2 mast cells was found. This interaction was most probably dependent on specific mediators released by both cell types since no adhesion of LAD2 cells to the SW756 cell monolayer was observed. On one hand, LAD2 cells increased the migration of cervical tumor cells, and on the other hand, SW756 cell supernatants evoked LAD2 cell degranulation, as shown by the increased release of β-hexosaminidase and histamine. Various morphological studies have shown degranulated mast cells located nearby neoplastic tissues (4, 48 – 50), suggesting that mast cells might be activated by tumor mediators or by direct cell-to-cell interactions.

To our knowledge, the present results are the first evidence of mast cell degranulation in response to incubation with the supernatant of a tumor cell line. Previous work by this group has shown that SW756 cells produce TNFα (38), which could be causing mast cell degranulation. Recent research has shown that TNFα enhances FcεRI-evoked mast cell degranulation by acting on the TNFα receptor 4-1BB (51). SCF is another possible mediator produced by SW756 cells that could account for mast cell degranulation (52). Cervical squamous epithelium and some cervical carcinoma cell lines exhibit an increased production of SCF (53). As recently reviewed, mast cell activity and survival are largely dependent on SCF, and it has been demonstrated that the LAD2 cell line expresses c-kit receptor (44). Further investigation of the putative mediators derived from cervical carcinoma cells that could regulate mast cell activation needs to be accomplished.

The coincubation experiments showed that SW756 cells evoked the release of histamine by LAD2 mast cells, which in turn contributed to increased SW756 cell migration. This was further demonstrated by the inhibition of this effect by pyrilamine, also known as mepyramine, the referent selective and high affinity H1R antagonist (11). Therefore, the results suggest that mast cell-derived histamine is a positive modulator of

SW756 cell migration by acting on H1R receptors. Similarly, it has been shown in HUVEC cells that H1R activation is a promoter of cell migration by a mechanism involving stress fiber formation and actin cytoskeleton reorganization (54).

In this study, a pharmacological approach to analyze histamine receptor function and its role on SW756 cell migration rate was assessed by the addition of specific receptor antagonists and histamine. Higher than physiological levels of histamine were required to characterize the histamine receptors involved in SW756 migration. This may be explained by the fact that in physiological conditions mast cells release a plethora of mediators that potentiate and overlap each other, which is not the case when the sole effect of histamine was being analyzed (55, 56). The results showed that histamine, acting on H1R and H4R, modulates the migration rate of SW756 cells in a concentration dependent manner. Unexpectedly, when histamine or pyrilamine were added alone to the wounded SW756 cell monolayer, no effect on SW756 cell migration rate was observed, suggesting that SW756 cells express H1R but do not produce histamine, as it was found in the present experiments. However, when histamine and pyrilamine were administered simultaneously, SW756 migration rate was significantly reduced. Since pyrilamine antagonizes histamine effects at H1R, the suggestion is that H1R activation increases SW756 cells migration rate. The inverse situation was observed when the effect of the H3R/H4R antagonist thioperamide was analyzed since the addition of this drug increased SW756 migration rate, but only in the presence of histamine. Therefore, by acting on H4R, histamine could exert an inhibitory effect on SW756 cell migration rate.

From the RT-PCR analyses it was found that the cervical carcinoma cell line SW756 expressed H1R and H2R mRNAs independently of the presence of serum in the culture media; however, H4R was only present when the cells were incubated with serum free media (L-15 SFM). Since H4R activation inhibited cell migration, it may be possible that H4R could be effectively blocking SW756 cell migration in response to histamine in a starved culture medium. Since the tumor microenvironment is an important factor for cancer progression and malignancy (42), differential expression of H4R depending upon media conditions could be an additional regulatory mechanism of neoplastic cell evolution that should be further analyzed. On the other hand, the lack of effect of ranitidine on SW756 migration rate suggests that the mRNA for H2R receptor may not be translated into a functional protein or may not play a role on SW756 cell motility.

The pharmacological analyses on the effect of cannabinoids on migratory potential of SW756 cells were performed with the endocannabinoid full agonist 2AG and the synthetic cannabinoid Win 55,212-2 (32). Both agonists inhibited SW756 cell migration rate, an effect that was reverted by the CB1-specific antagonist AM251, but not by the CB2-specific antagonist SR144528. This suggests a CB1-receptor specific mechanism on the action of cannabinoids on SW756 cell migration, which agrees with results previously reported for SW480 cells, a human colon cancer cell line (33). The fact that CB1 and CB2 receptors were involved in cannabinoid inhibition of SW756 cell migration rate evoked by mast cells or their supernatants could be explained by the results showing that CB2 receptors inhibited LAD2 mast cell degranulation. Therefore, cannabinoids may have the potential to inhibit mast cell-mediated increase in SW756 migratory rate by acting directly on their CB1 receptors or by acting indirectly through mast cell inhibition via CB2 receptors. Therefore, the inhibition of cervical carcinoma cell migration by cannabinoids may reinforce the already known antitumoral effects of these compounds (30) and encourages the development of potential therapeutic applications in cervical cancer cells (57).

Interestingly, LAD2 mast cells express mRNAs for CB2 and the four HR types (1 through 4), however the present experiments showed that only CB2 receptors were functionally capable of inhibiting LAD2 cell degranulation evoked by the SW756 supernatant. The significance of these findings should be further analyzed in future studies related to the autologous effect of mast cell mediators on their own function and phenotype.

The functional significance of immune cell infiltration of a tumor, specifically of mast cells located at the periphery of several neoplasias, is still a matter of controversy. Mast cell activation results in the expression of several mediators with diverse and sometimes opposite biological effects. The results of this study confirm a dual function showing mast cells regulating SW756 cell migration rate through histamine and cannabinoids. Histamine acting via H1R in cervical cancer cells could be pro-migratory, but when acting via H4R could inhibit migration, similar to the effect of cannabinoids through the activation of CB1 receptors. On the other hand, these results also showed that cervical carcinoma cell mediators can activate mast cells to degranulate, demonstrating an active and dynamic cross-talk between tumor cells and infiltrating mast cells as shown in morphologic studies of neoplastic tissues.

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