Histamine and Breast Cancer: A New Role for a Well Known Amine

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1. Introduction

Metastases are the most devastating aspect of cancer since most deaths from cancer are related to them. The ability of tumors to invade the neighboring extracellular matrix, which is primarily accompanied by augmented matrix metalloproteinases (MMPs) production and cell migration, is critical for metastases.

After surgical removal of primary breast tumors, malignant cells may still remain and radiotherapy is an efficient modality to reduce the risk of local recurrence. However, proliferative, invasive, and metastatic capacities can be increased in the surviving tumor cells of irradiated breast and other neoplasias (Baluna et al, 2006; Tsukamoto et al, 2007; Tsutsumi et al, 2009). To improve the efficacy of radiotherapy, this phenomenon must be further studied to elaborate therapeutic modalities to prevent radiation enhancement of cancer cell invasion.

Histamine is an endogenous biogenic amine extensively distributed throughout the organism which exerts multiple functions in physiologic and pathophysiological processes by stimulation of four G-protein coupled receptors (H1, H2, H3 and H4 histamine receptors) with different tissue expression patterns and functions. It is well known that diverse tumoral tissues and cell lines express the different histamine receptors through which histamine brings about its effects on cell proliferation, differentiation, survival and death. A great deal of evidence shows a relevant role of histamine in tumor progression, however controversial results are published depending on the cell type and the histamine receptor subtype that is activated (Blaya et al, 2010; Francis et al, 2009; Parsons & Ganellin, 2006; Soule et al, 2010). It has also been determined that numerous tumour tissues and cell lines express L-histidine decarboxylase, the histamine-synthesizing enzyme, and contain high levels of endogenous histamine which released to the extracellular media may exert its effects via a paracrine or autocrine regulation (Falus et al, 2001; Pós et al, 2004; Rivera et al, 2000). Additionally some effects on tumor growth may be mediated by histamine regulation of angiogenesis and immunity (Lázár-Molnár et al, 2002; Tomita et al, 2003).

Our research team has demonstrated the expression of histamine membrane receptors and their association to different signalling pathways in breast cancer biopsies and a large number of transformed cell lines, being our works the first ones to report the presence of H3

and H4 receptors linked to cell proliferation in breast and pancreatic cancer cell lines. We determined that histamine can modulate not only cell proliferation but differentiation, apoptosis and secretion of different growth factors (Cricco et al, 2006, 2008; Medina et al, 2006, 2008, 2009; Rivera et al, 2000, 2004). We have demonstrated that the highly invasive and metastatic breast cancer cell line MDA-MB 231 expresses the four known types of histamine receptors through which histamine differentially regulates cell proliferation in a dose dependent way. At concentrations over 10 μM a significant decrease in clonogenic growth was observed, while at concentrations lower than 0.5 μM proliferation was increased. The negative effect on proliferation was associated with the induction of arrest in G2/M phase of cell cycle, differentiation and a significant augmentation of apoptotic death (Medina et al, 2006).

Histamine and antihistamines have a modulatory effect on epithelial and endothelial cell adhesion and on the expression of different MMPs (Asano et al, 2004; Ciprandi et al, 2003; Gschwandtner et al, 2008). It has also been described a stimulatory action on migration of fibroblasts, haematopoyetic and immune cells, mainly via H4 receptors (Gschwandtner et al, 2010; Kohyama et al, 2010). However most of these reports refer to normal cells, existing less information regarding to tumor cells. Recently we have demonstrated the ability of HA to modulate MMPs, tissue inhibitors of matrix metalloproteinases (TIMPs) and cell adhesion in the breast cancer cell lines HBL-100 and MDA-MB 231 (Genre et al, 2009). We have also determined the upregulation of MMP2 and cell migration in the pancreatic carcinoma cell line PANC-1 by histamine (Cricco et al, 2006).

Reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H2O2) are continuously produced as by-products of aerobic metabolism primarily in the mitochondria and other cellular sources. In addition, external agents like ultraviolet radiation and ionizing radiation also increase intracellular ROS levels. Under physiologic conditions cells are protected against oxidative stress by an interacting network of antioxidant enzymes like superoxide dismutases, catalase and glutathion peroxidase. Though ROS may induce cellular damage when there is an imbalance between their generation and scavenging, numerous studies support the role of ROS as essential participants in cell signaling depending on their concentration, timing and location (Thannickal and Fanburg, 2000).

The aim of the present study was to investigate the role of HA and HA receptors activation in early cellular events involved in metastatic capacity, such as expression and activity of matrix metalloproteinases MMP2 and MMP9, cell migration and invasion in MDA-MB 231 cells. Since ionizing radiation may affect metastatic competence depending upon cell type and irradiation characteristics, and given that histamine increases MDA-MB 231 cells intrinsic sensibility to ionizing radiation by downregulating catalase activity and enhancing H2O2 intracellular levels at the same doses that inhibit cell proliferation (Medina et al, 2006), the possible interaction between histamine treatment and irradiation was also evaluated. The identification of drugs that can both regulate tumor cell survival and metastatic ability will help to delineate more effective strategies for therapeutic intervention in malignant diseases.

2. Effects of histamine and histamine receptor agonists upon matrix metalloproteinases MMP2 and MMP9

MMP2 and MMP9, also called gelatinase A and gelatinase B respectively, belong to a large family of zinc-dependent endopeptidases formed by more than 20 members which

participate in the proteolysis of basement membrane and extracellular matrix proteins, collagen and fibronectin. MMPs are involved in normal tissue remodeling events like wound healing, bone resorption and mammary involution. Their anomalous expression is associated to various pathological processes including inflammation and cancer (Freije et al, 2003). MMPs are secreted in a latent zymogen form (proenzyme). The inactive enzyme conformation is maintained due to thiol interactions between cysteine residues in the prodomain and the zinc atom present in the catalytic site of all MMPs. *In vitro*, MMPs activation can occur when the prodomain is cleaved by other proteases or when the zinc-cysteine bound is interrupted (Van Wart & Birkedal-Hansen, 1990).

Regulation of MMPs occurs on three levels: alteration of gene expression, activation of latent zymogens and inhibition by TIMPs. This regulation is tightly controlled in normal states while it is altered during tumor cell progression, being a critical step for cancer invasion and metastases (Chakraborti et al, 2003). Initially, it was believed that the major role of MMPs in metastases was to facilitate the breakdown of physical barriers. At present, it is widely accepted that MMPs may have a more complex role making important contributions at other steps in the metastatic process (Chabottaux & Noel, 2007). *In vitro* and *in vivo* studies demonstrate an entire correlation between MMP2, MMP9 and TIMPs expression and metastasis (Baum et al, 2007; Chambers & Matrisian, 1997; Duffy et al, 2008; John & Tuszynski, 2001; Wroblewski et al, 2002). Accordingly, MMP2 and MMP9 have been associated with breast cancer development and tumor progression (Köhrmann et al, 2009). An elevated activity of gelatinases in plasma and sera of patients with breast cancer confirms the role of these enzymes in the development of such tumors though clinical correlation with parameters like tumor grade and stage, size and lymph node involvement is still fragmentary (Decokc et al 2005; La Rocca et al, 2004; Stankovic et al, 2010).

In a previous work we determined a dissimilar role of histamine in the modulation of gelatinolytic activities and cell adhesion in mammary cell lines with different tumorigenic capacity (Genre et al, 2009), To further investigate histamine action and also histamine receptors implicated in breast cancer invasiveness we conducted *in vitro* experiments employing different doses of histamine and histamine receptor agonists and antagonists (listed in Table 1) in MDA-MB 231 cell line (ATCC HTB-26). Cells were cultured in RPMI supplemented with 10% fetal bovine serum, L-glutamine (0.3 g/L) and gentamicin (0.04 g/L) under standard conditions of 5% CO_2 and temperature of 37°C.

Ligand	Characteristic	References
2-(3-trifluoromethylphenil)histamine dimaleate (Sigma Aldrich)	H1 histamine receptor agonist	Leschke et al (1995). Synthesis and histamine H1 receptor agonist activity of a series of 2-phenylhistamines, 2-heteroarylhistamines, and analogues. *J Med Chem,* Vol. 38, No8, (April 1995), pp. 1287-1294, ISSN 0022-2623
Amthamine dihydrobromide (Tocris)	H2 histamine receptor agonist	Eriks el al (1992). Histamine H2 -receptor agonists -synthesis, in vitro pharmacology, and qualitative structure activity relationships of substituted 4-(2-Aminoethyl)thiazoles and 5-(2-Aminoethyl)thiazoles. *J Med Chem,* Vol. 35, No17, (August 1992), pp. 3239-3246, ISSN:0022-2623

Table 1. Histamine receptor agonists and antagonists employed in this study. (Continued)

Ligand	Characteristic	References
R(alpha) methylhistamine dihydrobromide (Tocris)	H3 histamine receptor agonist	Leurs el al (1995). The medicinal chemistry and therapeutic potential of ligands for the histamine H3 receptor. *Prog Drug Res,* Vol. 45, pp.107-165, ISSN 0071-786X
VUF 8430 dihydrobromide (Tocris)	H4 histamine receptor agonist	Lim et al (2006). Discovery of S-(2-guanidylethyl)-isothiourea (VUF 8430) as a potent nonimidazole histamine H4 receptor agonist. *J Med Chem*, Vol.49, No.23, (November 2006), pp. 6650-6651, ISSN 0022-2623
Clobenpropit dihydrobromide (Tocris)	H4 histamine receptor agonist and H3 histamine receptor antagonist	Liu et al (2001). Cloning and pharmacological characterization of a fourth histamine receptor (H_4) expressed in bone marrow. *Mol Pharmacol,* Vol.59, No3, (March 2001), pp 420-426, ISSN 0026-895X
Ranitidine hydrochloride (Sigma Aldrich)	H2 histamine receptor antagonist	Brittain & Daly (1981) A review of the animal pharmacology of ranitidine -a new, selective histamine H_2-antagonist. *Scand J Gastroenterol, Suppl*, Vol.69, (June 1981), pp. 1-9, ISSN 0085-5928
JNJ7777120 (Johnson & Johnson)	H4 histamine receptor antagonist	Jablanowski et al (2003). The first potent and selective non-imidazole human histamine H_4 receptor antagonists. *J Med Chem*, Vol. 6, No19, (September 2003), pp. 3957-3960. Sep 11;46(19):3957-60, ISSN 0022-2623

Table 1. Histamine receptor agonists and antagonists employed in this study.

2.1 Evaluation of matrix metalloproteinases activity

MMP2 and MMP9 gelatinolytic activity was evaluated by zymography, a powerful electrophoretic technique for identifying proteolytic activity of enzymes separated in polyacrylamide gels under nonreducing conditions. Cells were cultured in serum-free RPMI medium for 24h in absence or presence of treatments. Conditioned media mixed with non-reducing buffer were electrophoresed in 7% SDS-polyacrylamide gel containing 1% of gelatin. Gels were then rinsed, incubated (48h at 37ºC) and subsequently stained (0.1% Coomasie Brilliant Blue). Activity of lytic bands was determined by densitometry (Image J 1.42q, NIH, US).

There was a biphasic effect when cells were cultured with histamine concentrations varying from 0.1 µM to 20 µM. A significant increase in the activity of MMP2 and MMP9 pro and active forms was observed with 0.5 µM histamine (low dose) while a dramatic reduction in the activity of MMP2 and MMP9 (pro and active forms) occurred with histamine over 10 µM (high doses) as shown in Figure 1.

Testing H1, H2, H3 and H4 histamine receptor agonists we could observe that activities of both MMPs were differentially modulated. 10 µM H2 agonist amthamine evoked the reduction in lytic bands induced by high doses of histamine while 10 µM H4 agonists VUF 8430 and clobenpropit produced a significant rise of MMP2 and MMP9 activities as low doses of histamine did (Figure 2). The increase and decrease in gelatinolytic activities produced by histamine were blocked by the specific H4 antagonist JNJ7777120 (10 µM) and H2 antagonist ranitidine (10 µM) respectively (data not shown). In addition, no significant changes in both activities were determined employing H1 and H3 histamine agonists so that they were no longer tested in current assays.
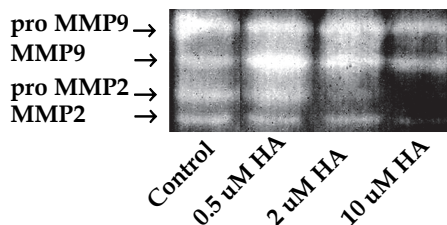
Fig. 1. Effect of histamine on MMP2 and MMP9 gelatinolytic activity. Activity was evaluated by zymography after 24h of treatment with different concentrations of histamine in serum free RPMI medium. The figure shows a representative gel.
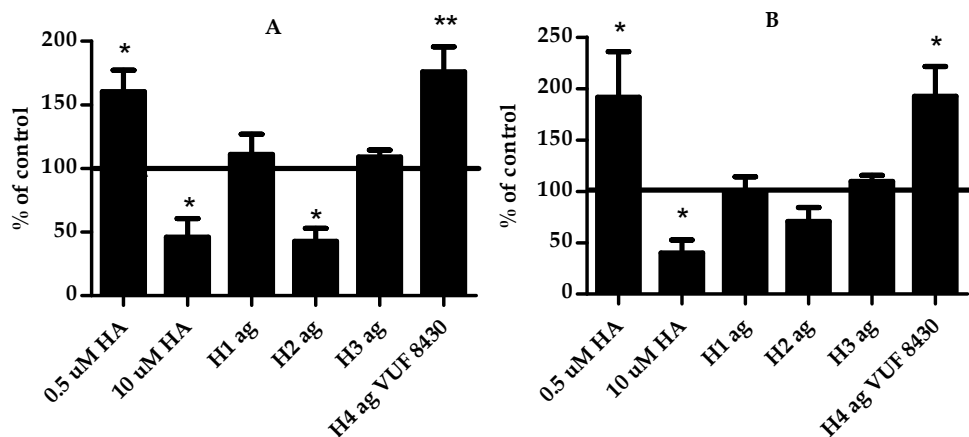

Fig. 2. Effect of histamine and histamine receptor agonists on MMPs gelatinolytic activity. A: MMP2 zymography. B: MMP9 zymography. Activity of lytic bands for each treatment was determined by densitometry and normalized to control values. Bars show the means ± SEM of three experiments run in triplicates. *$p < 0.05$ and **$p < 0.01$ vs control. One way Anova, Dunnet post test.

2.2 Determination of MMP2 and MMP9 mRNA steady state levels

MMP2 and MMP9 mRNA steady state levels were evaluated in cell cultures after 24h treatments using RT-PCR. Total RNA was extracted with Trizol®. Retrotranscription was carried out with MMLV enzyme. DNA was amplified by PCR and products were run in 2% agarose gels containing ethidium bromide. Semiquantification using GAPDH as housekeeping gene was performed with the Image J 1.42q (NIH, US) software.

	Primer sequence 5´-3´	PCR product size (bp)
MMP2	s ACC CAT TTA CAC CTA CAC CAA G a GTA TAC CGC ATC AAT CTT TTC CG	306
MMP9	s CCC ATT TCG ACG ATG AC a GGC ACT GAG GAA GAA TGA TCT AAG	639
GAPDH	s GCA GGG GGG AGC CAA AAG GG a TGC AGC CCC AG CGT CAA AG	566

Table 2. Sequence of primers used in PCR reactions.

A significant reduction of mRNA steady states levels of both MMPs was observed when MDA-MB 231 cells were treated with histamine concentrations over 10 μM in agreement to our previous report (Genre et al, 2009), being this effect reproduced by 10 μM amthamine. On the other hand, there was an important increase in mRNA expression when these cells received 0.5 μM HA (low dose of HA) or H4 agonist VUF 8430 (Figure 3). Similar results were obtained employing the H4 agonist clobenpropit.
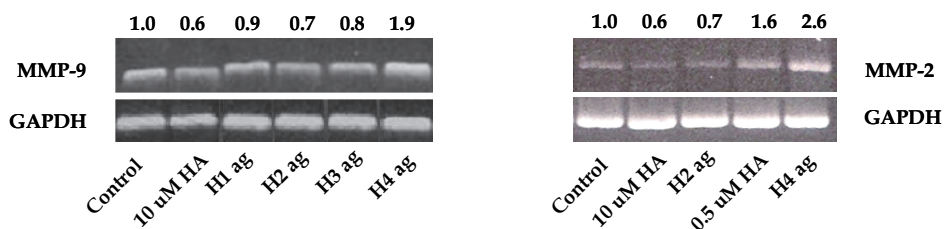

Fig. 3. mRNA expression of MMPs in MDA-MB231 cells. MMP2 and MMP9 mRNA steady state levels were determined by RT-PCR after 24h treatment with histamine and histamine receptor agonists. Figure shows representative gels for PCR products. The numbers above represent for each treatment the relative expression to controls of MMPs normalized to GAPDH.

2.3 Immunodetection of MMP2 and MMP9

Protein expression determination was performed by immunocytochemistry. MDA-MB 231 cells were grown on coverglasses and after 24h treatment cells were fixed, permeabilized and endogenous peroxidase was inhibited. Cells were incubated for 18h at 4°C with anti-MMP9 Ab (1/50, Calbiochem) or anti-MMP2 Ab (1/50, Cell Signalling) and for 2h with horse radish peroxidase conjugated anti-rabbit IgG (1/100, Sigma-Aldrich). This was followed by diaminobencidine and hematoxylin staining and observation by optical microscope.

Immunocytochemical staining supported the results of RT-PCR. A higher cytoplasmatic expression of MMP9 was seen when cells were treated with 0.5 μM histamine or H4 agonists. In turn a lower expression of MMP9 was detected in the presence of 10 μM histamine or H2 agonist (Figure 4). Similar results were obtained for MMP2 (data not shown).
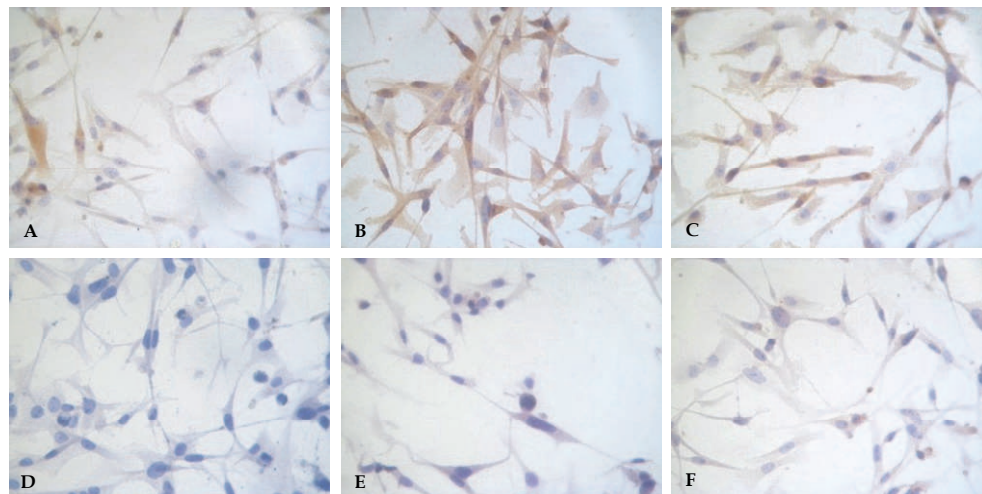
Fig. 4. Action of histamine and histamine receptor agonists on MMP2 and MMP9 protein expression in MDA-MB 231 cells. Expression was evaluated by immunocytochemistry. A: control, B: 0.5 μM HA, C: H4 agonist VUF 8340, D: negative control, E: 10 μM HA, F: H2 agonist. Photographs correspond to MMP9 expression. 400x magnification. Similar results were obtained for MMP2 expression.

Our research indicates that the mRNA and protein expression patterns of MMP2 and MMP9 were dose-dependently modified by HA through H2 and H4 receptors in MDA-MB 231 cells. Additionally, changes in gelatinolytic activity of MMP2 and MMP9 were registered in parallel to these results suggesting that an increment in protein expression may account for the increase in gelatinolytic activity.

These data constitute the first report about histamine-induced MMPs enhancement through H4 receptors in breast tumor cells. Most of *in vitro* reports upon histamine and MMPs modulation are related to inflammatory and allergic processes via H1 and H2 receptors and more recently via H4 receptors. Asano and coworkers showed that fexofenadine hydrochloride, a selective H1 receptor antagonist, may inhibit mRNA and protein expression of MMP2 and MMP9 in nasal fibroblasts and thereby possibly reduce the severity of allergic rhinitis, characterized by remodeling of the nasal wall and eosinophil and mast cell infiltration (Asano et al, 2004). It has been reported the histamine-induced production of MMP2 through H1 and H2 receptors in microvascular endothelial cells is associated to angiogenic processes (Doyle & Haas, 2009). An increase in mRNA level, protein expression and gelatinolytic activity of MMP9 is observed in human keratinocytes via H1 receptors stimulation in skin remodelling and fibrosis (Gschwandtner et al, 2008). Moreover, the *in vitro* production of other MMPs as MMP3, MMP8 and MMP13 is stimulated by histamine in human articular chondrocytes and rheumatoid synovial fibroblasts (Tetlow & Woolley, 2002; 2004). Modulation of mRNA for MMP1 by histamine has also been described in human corneal epithelial cells (Sharif et al, 1998). Regarding tumor cells, we have reported that histamine is able to reduce cell adhesion and to enhance the gelatinolytic activity of MMP2 in the pancreatic cancer cell line PANC-1 (Cricco et al, 2006). Furthermore, tranilast an antiallergic compound used clinically to control atopic and

fibrotic disorders exerts its action by reducing the expression and activity of MMP2 in human malignant glioma cells (Platten et al, 2001).

3. Histamine and histamine agonists modulate MDA-MB 231 cells migration and invasion

In view of current results on MMP2 and MMP9 expression and activity we explored the migratory capacity of MDA-MB 231 mammary cells. Directed migration is an essential component of cell invasion during tumor progression and metastasis. The migratory response of MDA-MB 231 cells to histamine and histamine agonists was investigated using a 24-well transwell unit with polyethylene terephthalate (PET) membranes having a pore size of 8.0 µm (BD Falcon, Basel, Switzerland). For invasion assay the upper part of the transwell was coated with Matrigel®, a synthetic basement membrane (BD Biosciences, Bedford, MA). A fixed number of cells (1.10^4/chamber) was plated on the transwell. The medium in compartments of the lower chambers contained 2% FBS plus treatments. Inserts were incubated for 6 h at 37°C. Non migrating cells on the upper surface of membranes were gently scrubbed with a cotton swab. Cells migrated or invading the lower surface of the membrane were fixed with methanol, and stained with hematoxilin. Ten random fields were counted under a light microscope at 50x magnification.

Histamine exerted a dual action on the migratory behavior of cells. It is improved by doses of histamine lower than 1 µM and reduced by concentrations over 10 µM (Figure 5). The stimulatory response disclosed by histamine was evoked by the H4 agonists and prevented by the specific H4 antagonist. On the other hand, histamine-induced inhibitory effect on cell motility was mimicked by the H2 agonist while relapsed by ranitidine. H1 agonist and H3 agonist did not modify cell migration; consequently they were not longer tested.

In addition similar results were displayed by histamine and histamine agonists when invasiveness was evaluated, as depicted in Figure 6. These results are in full concordance with those obtained for MMPs, reinforcing the idea that histamine may have a key role as mediator of MDA-MB 231 cells invasive ability.

A body of evidence shows a chemotactic response of inflammatory and immune cells to histamine. Because of its preferential expression in immunocompetent cells, the H4 receptor is closely related with the regulatory functions of histamine during the immune response. It has been published that histamine mediates chemotaxis via the H4 receptor in mast cells, in human monocyte-derived dendritic cells and in eosinophils, related to immune and inflammatory disorders (Baumer et al, 2008; Gutzmer et al, 2005; Hofstra et al, 2003; Ling et al, 2004; Thurmond et al, 2004). Barnad et al (2007) proved that exposure to histamine cause eosinophils to migrate from the bloodstream to the inflammatory focus into tissues, inducing actin polymerization through the H4 receptor. Histamine also activates signaling pathways typical of chemotaxis inducing migratory responses in T lymphocytes, via the H4 receptor (Truta-Feles K et al, 2010). Besides this chemotactic action in immune cells it has also been reported that histamine promotes invasiveness specifically through activation of H1 receptor in human cytotrophoblasts required to initiate blastocyst implantation (Liu et al, 2004).

Regarding tumor cells it has been reported that histamine acts as a chemoattractant for human carcinoma and melanoma cells via H1 receptor stimulation (Blaya et al, 2010; Tilly et al, 1990). Accordingly, the antiallergic compound tranilast inhibits cell migration and invasion in human malignant glioma cells blocking H1 receptors (Platten et al, 2001). A

divergent response (stimulatory or inhibitory) on the migration rate of SW756 cervical carcinoma cells has been disclosed by histamine acting on H1 receptor or H4 receptor, respectively (Rudolph et al, 2008). However, as far as we know, our work is the first report about histamine-modulated migration and invasion through H4 and H2 receptors in breast tumor cells.
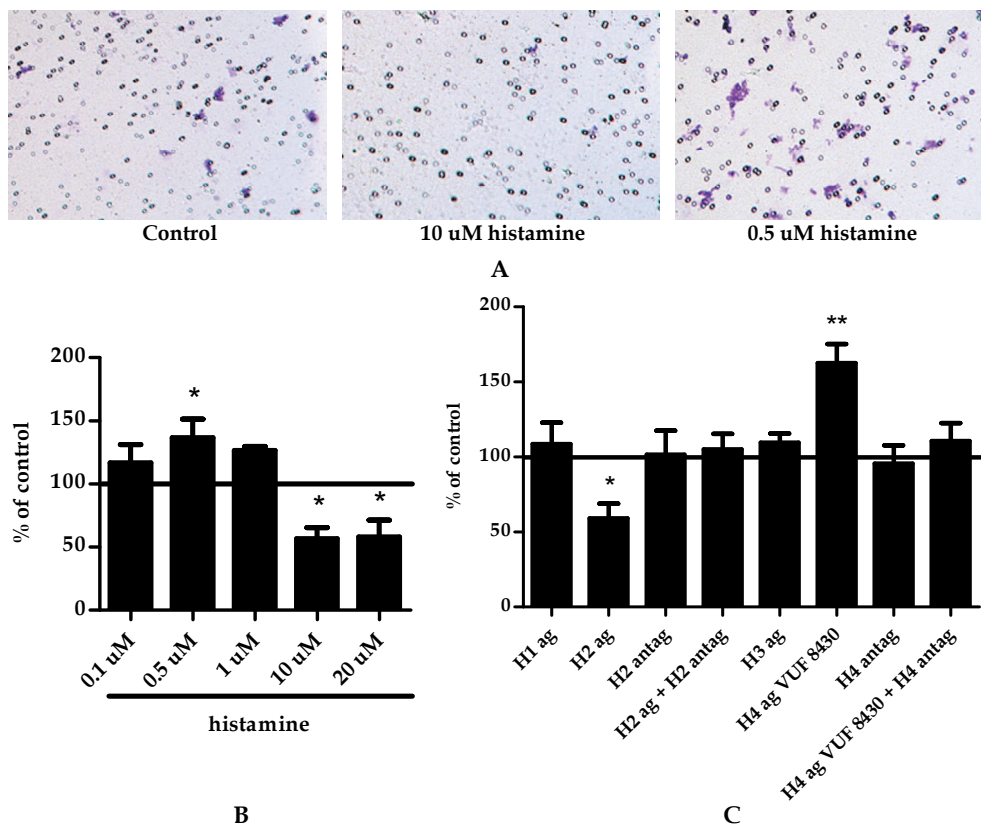

Fig. 5. Histamine induced migration of MDA-MB 231 cells. Migration was evaluated by using transwell units with 8 μm pore size PET membranes. A: Photographs are representative of random fields observed at 50x magnification. B-C: Bars show the means ± SEM of three experiments run in duplicates. Results are expressed for each treatment as the number of cells on the lower side of membranes normalized to control values. B: Concentration-response curve of histamine. C: Effect of histamine receptor agonists and antagonists on cell migration. *p<0.05 and **p<0.01 vs control. One way ANOVA, Dunnet post test.

4. H2O2 involvement in histamine actions

Many cell surface receptors produce transient levels of ROS (specifically H_2O_2) when are activated by peptide growth factors, cytokines and ligands of G protein-coupled receptors
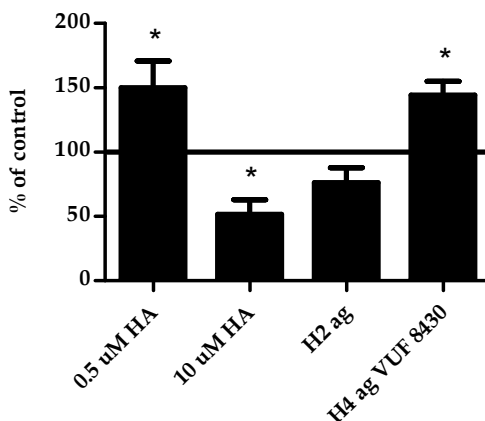
Fig. 6. Effect of histamine and histamine receptor agonists on cell invasion. Invasion was evaluated by using transwell units with 8 µm pore size PET membranes coated with Matrigel®. Bars show the means ± SEM of three experiments run in duplicates. Results are expressed for each treatment as the number of cells invading the lower side of membranes normalized to control values. *p<0.05 vs control. One way Anova, Dunnet post test.

(GPCRs) like histamine receptors (Rhee et al, 2000). In a previous work we reported that high doses of histamine enhance H2O2 intracellular levels in MDA-MB 231 cells (Medina et al, 2006).

ROS will contribute to various aspects of malignant tumors, including carcinogenesis, aberrant growth and metastasis which is a complex process including epithelial-mesenchymal transition (EMT), migration, invasion of the tumor cells and angiogenesis around the tumor lesion (Pani et al, 2009). Significant amounts of ROS are able to kill cancer cells through their oxidative action. At lower concentrations, however, ROS work as second messengers in signal transduction, and activate a variety of proteins or upregulate their transcription. The ROS-regulated genes relevant to EMT and metastasis include EGF, EGFR, VEGF, E-cadherin, integrin and MMPs (Nishikawa 2008; Wu, 2006).

In order to explore the effect of ROS on cellular events related to invasiveness of MDA-MB 231 cells we conducted the following experiments.

4.1 Measurement of ROS levels

The fluorescent dye dichlorofluorescein diacetate (DFH-DA) is an important tool to indicate oxidations in cells and is one of a very few markers available for measuring intracellular ROS levels in live cells. DFH-DA is a non polar and non fluorescent compound that freely diffuses through cell membranes. Once it enters the cells, its ester moiety is cleaved by intracellular esterases, and then DFH is retained in the cells. Oxidation of DFH by ROS yields a fluorescent species that can be detected by flow cytometry.

In order to investigate the effect of ROS on cellular events related to invasiveness we measured the steady state levels of ROS employing 5 µM DFH-DA dye in MDA-MB 231 cells after 1h treatment with histamine, histamine agonists and antagonists. Exogenous catalase (125 IU/mL) was added 15 minutes before treatments to metabolize intracellular H2O2 levels.

Histamine augmented intracellular steady state of ROS levels in a dose-dependent manner (Tabla 3). Histamine in low concentration and 10 μM H4 agonists increased intracellular ROS levels up to 135%, while high doses of histamine and 10 μM H2 agonists augmented them up to 190%. When exogenous catalase was added to histamine-treated cells there was a significant reduction of ROS levels almost to control value confirming that intracellular H_2O_2 was the major species involved since H_2O_2 is the only substrate for catalase.

Treatment	mean fluorescence (% respect of control values)
0.5 μM HA	123 ± 5
5 μM HA	140 ± 8*
20 μM HA	184 ± 10**
H4 agonist	135 ± 9*
H2 agonist	190 ± 15**
catalase	83 ± 6
catalase + 10 μM HA	120 ± 7
catalase + 0.5 μM HA	105 ± 6

Table 3. Effect of histamine, histamine receptor agonists and catalase on ROS levels in MDA-MB 231 cells. ROS levels were assessed by flow cytometry using DFH-DA, a specific fluorescent dye, and mean fluorescence values for each treatment were normalized to controls. Table show the means ± SEM of three experiments run in duplicates *p<0.05 and **p<0.01 vs control. One way Anova, Dunnet post test.

4.2 Gelatinolytic activity is modified by endogenous and exogenous H2O2 levels

MDA-MB 231 cells were treated with different concentrations of exogenous H_2O_2 and assayed by zymography. A biphasic response of MMP2 and MMP9 was observed depending on the H_2O_2 concentration added, as shown in Figure 7. Results indicate that different thresholds of H_2O_2 may be required for enhancing or inhibiting MMPs activities.

A link between ROS and MMPs has been widely discussed. Evidence supports that activation of proenzymes (MMP1, MMP2, MMP7 and MMP9) is regulated by ROS through interactions with thiol groups (Nelson & Melendez, 2004). It has been reported that higher doses of H_2O_2 may alter signal transduction pathways leading to protein degradation (Rhee et al, 2003). Our data are in agreement with Rajagopalan and coworkers who demonstrated ROS-induced activation of MMP2 at low doses of exogenous H_2O_2 (4 μM) and inactivation at higher doses (10–50 μM) in macrophage-derived foam cells when they studied the stability of atherosclerotic plaques (Rajagopalan et al, 1996). Suppression of MMP2 activity by H_2O_2 in a dose- and time-dependent manner was also observed during acute ulceration being reverted by antioxidants (Ganguly et al, 2006). However, most of reports inform about a positive modulation of MMP2 and MMP9 in response to oxidative stress in different tissues and cell lines including mammary cells (Mori et al, 2004).

Addition of exogenous catalase to tumor mammary cells half an hour before treatments was able to impede both the increase and decrease in gelatinolytic activities disclosed by

histamine and histamine agonists confirming that intracellular H2O2 levels were involved in the modulation of enzymatic activity of both MMPs (Figure 8).
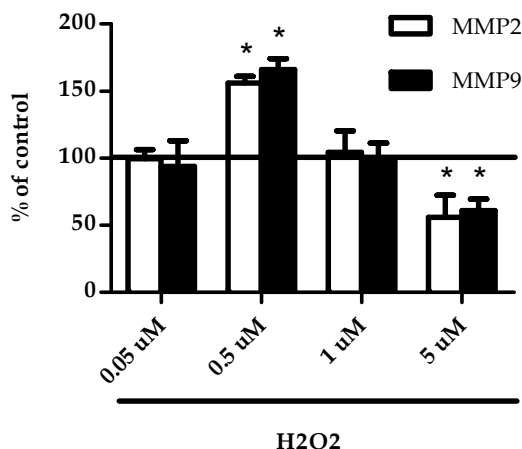

Fig. 7. Action of exogenous H2O2 on MMP2 and MMP9 gelatinolytic activity. Concentration-response curve obtained by zymography. Activity of lytic bands for each treatment was determined by densitometry and normalized to control values. Bars show the means ± SEM of three experiments run in triplicates *p<0.05 vs control. Two way Anova, Bonferroni post test.
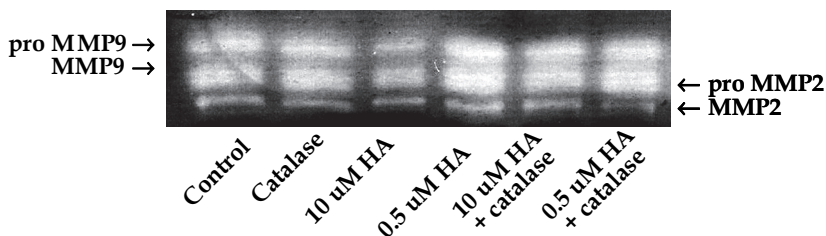

Fig. 8. Effect of catalase on histamine-regulated gelatinolytic activity of MMP2 and MMP9. Activity was evaluated by zymography after 24 h of treatment in serum free RPMI medium. Figure shows a representative gel.

4.3 Cell migration is influenced by H2O2 levels

In order to correlate H2O2 levels with the migratory capacity in MDA-MB 231 cells, we assayed different H2O2 concentrations using transwells. Cell migration was modified by exogenous H2O2 in a dose dependent manner (Figure 9A). It was significantly stimulated by low doses while inhibited by high ones. Considering that histamine and histamine agonists differentially modulate intracellular H2O2 as shown in table 3, our research suggests that the magnitude of these rises in histamine-treated cells may therefore be essential for the migratory and invasive behavior. This possibility is supported by the results obtained when exogenous catalase (125 IU/mL) was added to cell cultures (Figure 9B).
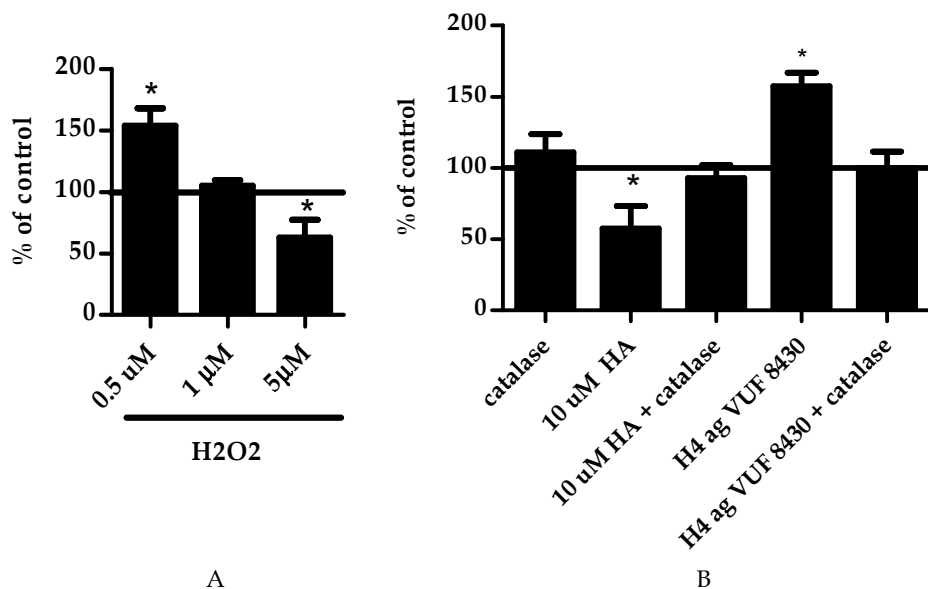
A   B

Fig. 9. Effect of H2O2 on migration of MDA-MB 231 cells. Migration was evaluated by using transwell units with 8 um pore size PET membranes. Bars show the means ± SEM of three experiments run in duplicates. Results are expressed for each treatment as the number of cells counted on the lower side of membranes normalized to control values. A: Concentration-response curve for exogenous H2O2. B: Effect of catalase on histamine induced-cell migration. *$p<0.05$ vs control. One way ANOVA, Dunnet post test.

Overall, our data strongly support the hypothesis that ROS are involved in histamine-induced modulation of MMP2 and MMP9, which are assumed to play a critical role in tumor invasion. Divergent migration and invasion responses (stimulatory or inhibitory) to histamine may be due to changes in the cellular redox balance through H2 and H4 receptor activation as in the case of MMPs. Likely, when H2O2 is produced at high concentrations in MDA-MB 231 cells, it may be capable to affect enzymatic activities by altering signaling pathways and even cause cellular damage. Many elements in the process leading to cell migration are considered to be redox-sensitive (Pani et al, 2009; Svineng et al, 2008). ROS modify the activity of several key enzymes, resulting in the reorganization of actin cytoskeleton, adhesion and stimulation of migration. There is evidence that ROS can regulate such critical target molecules as PKC, MAPK, PI3K, tyrosine phospatases, PTEN, Src and focal adhesion kinase (FAK) (Rhee et al, 2000, 2003).

Preliminary results obtained in our laboratory show an increase in c-Src phosphorylation in the presence of low doses of histamine or H2O2 exogenously added suggesting a possible role of this kinase in MDA-MB 231 cells migration induced by histamine (data not shown).

4.4 Determination of catalase activity

We have previously reported that histamine in a high concentration inhibits catatase activity in MDA-MB 231 cells (Medina et al, 2006). To further correlate catalase activity with the intracellular H2O2 levels generated by histamine and histamine agonists treatments, we

measured the endogenous enzymatic activity spectrophotometrically. Briefly, cells were treated for 24h, scrapped and collected in potassium phosphate buffer 50 mM pH 7.0. This was followed by sonic disruption. Protein concentration was determined by Bradford assay. Catalase activity was determined using whole cell homogenates by measuring the exponential decay of 20 mM H2O2 ($\Delta\epsilon_{240}$ = 43.6 M^{-1} cm^{-1}) at 25°C monitored at 240 nm. Specific activity was expressed as the number of catalase units per mg of protein. One catalase unit is defined as the amount of catalase that breaks 1 µmol of H2O2 down per minute, at 25°C and pH 7.

There was a reduction of the enzymatic activity when cells were cultured in the presence of 0.5 µM histamine or H4 agonists, being this reduction more significant at higher doses of histamine or with H2 agonists (Figure 10). Data indicate that the modulation of catalase activity in our experimental conditions is critical in the control of endogenous H2O2 levels which in turn trigger different biological responses. In this sense, other authors have demonstrated in colon and liver tumor cells that high basal levels of ROS are mainly controlled by catalase (Laurent et al, 2005). Expression of catalase is known to be regulated at message, protein and activity levels. Soluble factors from tumor cells as TNF alpha have been suggested to be repressors of catalase expression, and cell signaling molecules as PKA, PKC and Casein Kinase II have been reported to elevate catalase activity *in vitro* (Nishikawa 2002; Reimer el 1994; Yano & Yano, 2002). Histamine could act at any of these levels (Baker et al, 2002; Igaz et al, 2001; Leurs et al, 2009; Steffel el al, 2006)

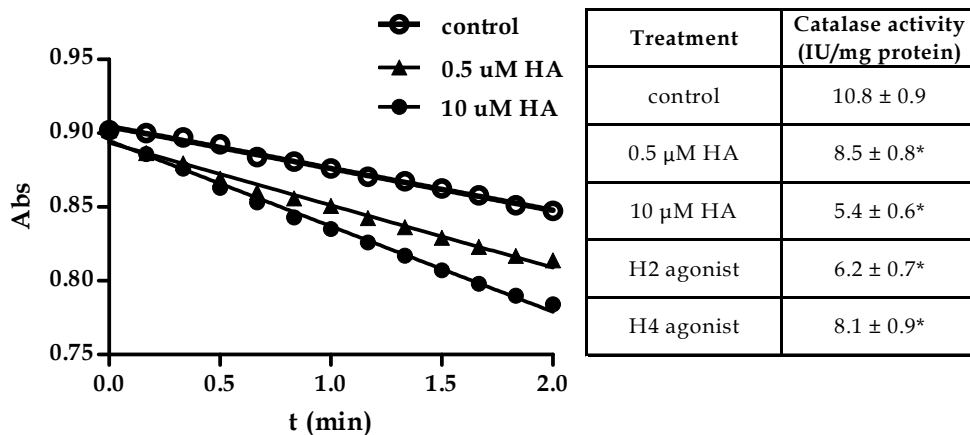| Treatment	Catalase activity (IU/mg protein)
control	10.8 ± 0.9
0.5 µM HA	8.5 ± 0.8*
10 µM HA	5.4 ± 0.6*
H2 agonist	6.2 ± 0.7*
H4 agonist	8.1 ± 0.9*

Fig. 10. Effect of histamine and histamine receptor agonists on catalase activity. Activity was evaluated spectrophotometrically by measuring the extinction of the H2O2, the substrate of catalase enzyme. Slopes of straight lines fit by linear regression represent the rate of extinction and are correlated with enzymatic activity. Embedded table shows catalase activities for histamine and histamine receptor agonists. Results are expressed as means ± SEM of two experiments run in duplicates. *p<0.05 vs control. One way Anova. Dunnet post test.

5. Ionizing radiation enhances the activity of MMPs and cell migration – Interaction between histamine and ionizing radiation

The mechanisms that control the therapeutic efficacy of radiotherapy have classically focused on the ability of ionizing radiation to kill cancer cells while sparing normal tissues. Radiation therapy after surgery consists typically of irradiation of the breast with 50-60 gray, delivered over 6 weeks in 2 gray fractions. Fractionation allows time to normal cells to recover, to tumor cells to reenter to radio-sensitive phase of the cell cycle and to hypoxic tumor areas to re-oxygenate and as a result become more responsive to ionizing radiation.

Sensitivity of tumor cells to ionizing radiation is crucial to outline the probability of local control and finally of cure of cancers by radiotherapy. Many factors are involved in affecting susceptibility of tumor cells to ionizing radiation and the generation of ROS as a result of photon irradiation accounts for approximately 75% of radiation-induced damage. ROS production occurs within seconds of starting radiation treatment and this initial redox perturbation has important implications in terms of the final cellular response to ionizing radiation.

As already mentioned we have previously demonstrated that histamine is able to increment MDA-MB 231 cells intrinsic radiosensitivity by downregulating catalase activity and increasing H2O2 intracellular levels at the same doses that inhibits cell proliferation. Recently we have established a radioprotective role of histamine on bone marrow against cellular damage induced by ionizing radiation (Medina et al, 2010). We have also proved that histamine prevents radiation-induced toxicity on small intestine by modulating the antioxidant enzymes expression and by suppressing apoptosis and increasing proliferation of damaged intestinal mucosa (Medina et al, 2007). Intracellular ROS concentration is critical for cell growth and survival; in normal cells ROS levels are kept low, but quite the opposite in tumor cells high levels of ROS close to the threshold of cytotoxicity are related to cell proliferation. Thus drugs that modulate antioxidant enzymes may differentially affect normal and neoplastic cells growth and death. In view of these antecedents, we proposed to study the interaction between histamine and ionizing radiation on the proinvasive ability of this cell line. MDA-MB 231 cells were gamma irradiated with a 2 gray dose using an IBL 437C, H type irradiator in presence or absence of histamine or histamine agonists. After irradiation culture media were conditioned for 24 h and then zymographies were carried out; migration assays were immediately performed. Ionizing radiation increased ROS levels in irradiated control cells and a rise in MMPs activity and cell migration was also found compared to non irradiated control cells (Figure 11). This effect observed on cellular events related to MDA-MB 231 invasiveness was counteracted when irradiated cells were treated with high doses of histamine or with H2 agonist, which may be due to the largest amount of ROS generated in these conditions.

The effects of irradiation on malignant biological behaviors of cells surviving irradiation have been reported for a variety of tumor cells. Many of these reports provide evidence that irradiated tumor cells acquire malignant potency through increased motility and invasiveness, up-regulation of MMPs, as well as an enhanced capacity for adhesion. The activation of different signal transduction cascades including stress kinases, the PI3K/Akt/NF-kB pathway or the c-Src is implicated in these radiation-induced responses. The same signaling mechanisms are engaged in ROS mediated actions on invasive capacity (Cheng el al, 2006; Hwang et al, 2006; Jung et al, 2007; Wild-Bold el al 2006). Current studies
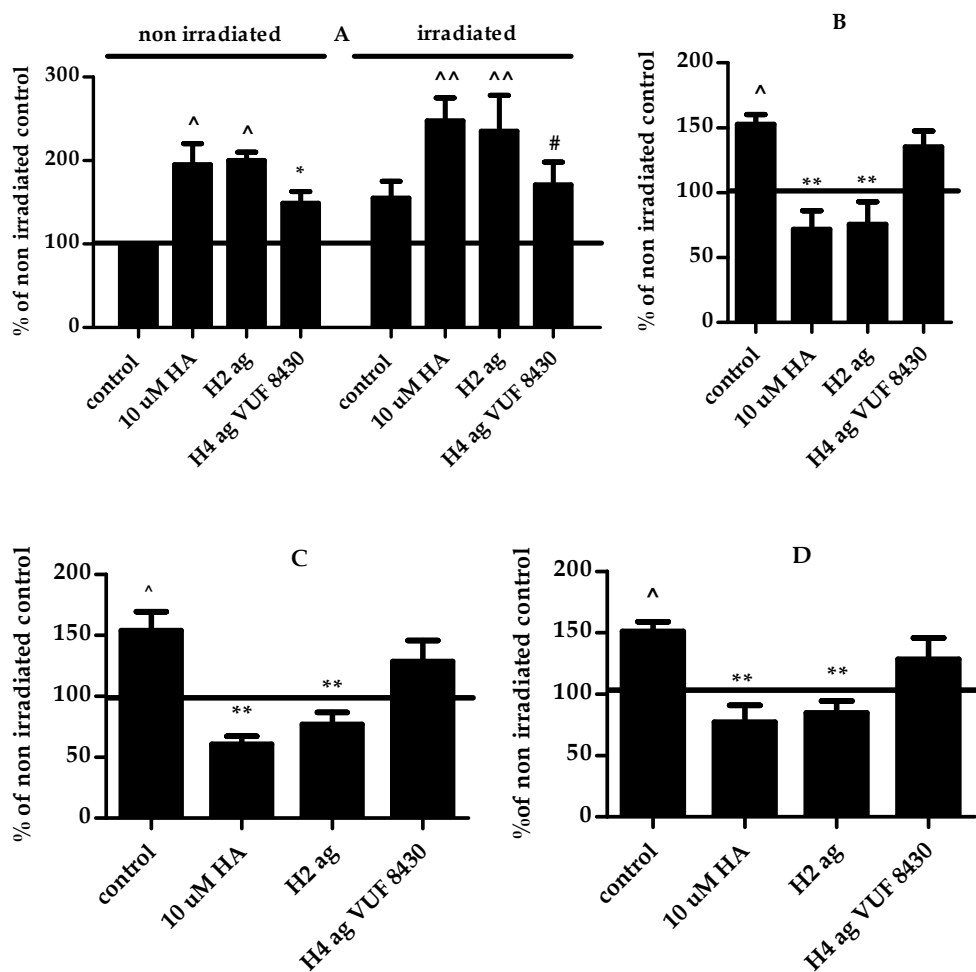
Fig. 11. Effect of ionizing radiation on MDA-MB 231 cells. A: ROS levels induced by histamine and histamine receptor agonists. B: Effect of histamine and histamine receptor agonists on cell migration of irradiated cells. C: Effect of histamine and histamine receptor agonists on MMP2 gelatinolytic activity of irradiated cells. D: Effect of histamine and histamine receptor agonists on MMP9 gelatinolytic activity of irradiated cells. Bars represent the means ± SEM of at least three experiments run in duplicates ^ $p<0.05$ and ^^$p<0.01$ vs non irradiated control, *$p<0.05$ vs 10 µM HA and vs control, **$p<0.01$ vs control, # $p<0.05$ vs 10 µM HA. Two way Anova, Bonferroni post test.

in our laboratory are intended to confirm the crucial role of distinct levels of ROS generated in the opposite responses of MDA-MB 231 cells to different histamine concentrations and ionizing radiation, and to identify the signaling pathways concerned.

6. Conclusions

Understanding the molecular mechanisms of metastases is one of the most relevant issues in cancer research. New growths (metastases) at distant sites from the primary tumor require a number of steps to be completed successfully. Tumor cells must bind to one or more components of the basement membrane or extracellular matrix, degrade them to migrate, intravasate in blood and lymphatic vessels and finally extravasate to be seeded at distant locations. MMP2 and MMP9 are mainly involved in these processes, being abundantly expressed in various malignant tumors.

In the last decade a subcutaneous formulation of histamine dihydrochloride has been used as an adjuvant with interleukin-2 therapy for the potential treatment of metastatic melanoma, acute myelogenous leukemia and renal cell carcinoma, mainly based upon its action on immunity (Perz & Ho, 2008). Our research provides novel evidence for a possible use of histamine as a pharmacological agent with low side effects that targets oncogenic pathways which may regulate breast tumor cell proliferation and/or survival and simultaneously may control invasion and metastasis.

Radiotherapy is a highly effective modality for locoregional treatment of breast tumors and other cancers. Despite the fact that it has been classically considered to exert its therapeutic effect by killing tumor cells, clinical and experimental evidence indicates that results extend beyond cancer cell death pointing out that ionizing radiation might promote a metastatic behavior of cancer cells. Our current results also open a perspective for the potential use of histamine to improve radiotherapy efficacy not only increasing intrinsic radiosensitivity of breast tumor cells specifically, but wielding an effect on the possible development of radio-induced metastases.

In view that a shift in the paradigm from a population-based to a personalized patient-based treatment emerges as a near step in cancer therapy correlating molecular expression signatures with treatment outcomes, the determination of histamine receptors in patient tumors seems feasible and may help design more effective therapies.

7. Aknowledgements

8. References

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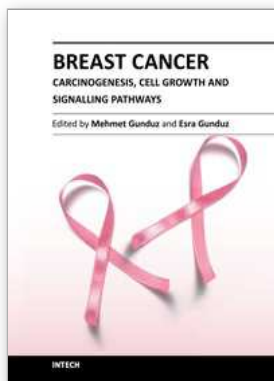
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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