Histamine and spontaneously released mast cell granules affect the cell growth of human hepatocellular carcinoma cells

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Abstract

The role of mast cells in tumor growth is still controversial. In this study we analyzed the effects of both histamine and pre-formed mediators spontaneously released by mast cells on the growth of two human hepatocellular carcinoma cell lines, HA22T/VGH and HuH-6, with different characteristics of differentiation, biological behavior and genetic defects. We showed that total mast cell releasate, exocytosed granules (granule remnants) and histamine reduced cell viability and proliferation in HuH-6 cells. In contrast, in HA22T/VGH cells granule remnants and histamine induced a weak but significant increase in cell growth. We showed that both cell lines expressed histamine receptors H_1 and H_2 and that the selective H_1 antagonist terfenadine reverted the histamine-induced inhibition of HuH-6 cell growth, whereas the selective H_2 antagonist ranitidine inhibited the histamine-induced cell growth of HA22T/VGH cells. We demonstrated that histamine down-regulated the expression of β-catenin, COX-2 and survivin in HuH-6 cells and that this was associated with caspase-3 activation and PARP cleavage. On the contrary, in HA22T/VGH cells expression of survivin and β-catenin increased after treatment with granule remnants and histamine. Overall, our results suggest that mediators stored in mast cell granules and histamine may affect the growth of liver cancer cells. However, mast cells and histamine may play different roles depending on the tumor cell features. Finally, these data suggest that histamine and histamine receptor agonists/ antagonists might be considered as "new therapeutic" drugs to inhibit liver tumor growth.

Keywords: β-catenin; BIRC5 protein, human; carcinoma, hepatocellular; cyclooxygenase 2; histamine; mast cells

Introduction

Mast cells (MC) are tissue-resident cells found widely distributed in the mucosa and subcutaneous connective tissue where they serve as the first line of defense against pathogens. MC are able to contribute to immunologic reactions and inflammation by producing mediators and cytokines. Indeed, depending on their subtypes MC may be activated by different stimuli which can be either immunologic or non-immunologic, such as neuropeptides, including substance P (SP) (Lowman et al., 1988). Activated MC may extrude granule-associated mediators, which can be pre-stored (i.e. histamine, serotonin, α-hexosaminidase, proteoglycans, and neutral protease), or novo-synthesized, (i.e. lipid-derived substances, and various cytokines, for example TNF-α and IL-6) (Gordon and Galli, 1990; Cocchiara et al., 1999a; Azzolina et al., 2003). MC numbers increased in association with malignant tumors such as colorectal carcinomas (Fisher et al., 1989), cervical (Graham and Graham, 1966) and breast cancer (Fisher et al., 1985), though the functions of the intra-tumor mast cells have not yet been clarified. Recently, mast cell accumulation has been shown both in liver (Terada and Matsunaga, 2000) and in gastric carcinoma (Jiang et al., 2002), suggesting a role for these cells in tumor immunology. There is also evidence that tumor-associated mast cell accumulation promotes tumor growth and metastasis in some conditions (Toth-Jakatics et al., 2000).

Histamine is the principal mediator stored in MC granules and may act as an autocrine growth factor in some tumor types, such as melanoma and colorectal carcinoma (Adams et al., 1994; Falus et al., 2001; Masini et al., 2005) and histamine receptors are also expressed in numerous malignant cell types. Many histamine receptor antagonists are being studied in clinical trials for cancer treatments

(Brandes *et al.*, 1994; Matsumoto *et al.*, 2002; Reyno *et al.*, 2005).

To date, little is known either about the relationship between the presence of activated mast cells and liver tumor growth, or about the role played by histamine released by mast cells in the progression of the disease. In a previous study, we investigated the presence of tryptase-positive mast cells in human hepatocellular carcinoma (HCC) tissues (Cervello *et al.*, 2005). HCC tissues with different histological grades showed different numbers of mast cells, with well-differentiated HCC showing the highest number. They tended to decrease in less-differentiated HCC, suggesting a possible role in the early stage of HCC development (Cervello *et al.*, 2005).

In the present study we investigated the role of histamine and mediators spontaneously released by mast cells on HCC cell growth. We showed that exocytosed granules (granule remnants) and histamine decreases cell growth in HuH-6 cells, possibly through the down-regulation of β-catenin, cyclooxygenase-2 (COX-2) and survivin expression, and the activation of apoptotic response, as shown by the induction of caspase-3 and nuclear poly (ADP-ribose) polymerase (PARP) cleavage. In contrast, granule remnants and histamine induce the up-regulation of β-catenin and survivin expression in HA22T/VGH cells, possibly promoting cell growth. Moreover, we showed that H_1 receptor antagonist terfenadine reverts the histamine-induced inhibition of cell growth in HuH-6 cells, whereas H_2 receptor antagonist ranitidine reverts histamine-induced cell growth in HA22T/VGH cells. Overall, histamine or histamine receptor agonists/antagonists might be considered as "new drugs" which are able to inhibit HCC growth.

Materials and Methods

Reagents

MEM, FCS, glutamine, penicillin, streptomycin, histamine, terfenadine and ranitidine were purchased from Sigma (Milano, Italy). The histamine antagonist receptors terfenadine and ranitidine were dissolved in DMSO and then diluted to give a final concentration of 0.05% DMSO. The following antibodies were used: rabbit polyclonal antibody against human COX-2 (Cayman Chemical, Ann Arbor, MI), rabbit polyclonal antibody against human survivin (Abcam Limited, Cambridge, UK), rabbit polyclonal antibody against human PARP (Cell Signaling Technology, UK), mouse monoclonal antibody against human β-catenin (Transduction Laboratories, Lexington, KY), rabbit polyclonal antibody against human caspase-3 (Santa Cruz Biotechnologies, Santa Cruz, CA), and mouse monoclonal antibody against human β-actin (Sigma-Aldrich Srl, Milan, Italy).

Preparation of peritoneal mast cells, granule remnants, and granule remnant-free supernatants

Peritoneal mast cells (PMC) were recovered from female Wistar rats as previously described (Cocchiara *et al.*, 1999b). Briefly, after peritoneal lavage with 10 ml Tyrode's buffer plus 0.03 g/l BSA, the mast cells were isolated on BSA gradient (38% w/v) centrifuged at $500 \times g$ for 15 min and the pellet was purified at 98%. The trypan blue-exclusion test indicated cell viability greater than 95%. 3×10^5 cells in 1 ml were plated in each well of a 24-well plate and cultured in serum-free medium at 37°C in 5% CO_2. After 24 h the culture medium was harvested and centrifuged for 10 min at $1,000 \times g$ to obtain total "mast cell releasate" (total), which contained all the material spontaneously released from mast cells. The releasate was subsequently centrifuged again for 10 min at $10,000 \times g$ to obtain a supernatant containing soluble mediators, the "granule remnant-free supernatant" (GR-FS) and a pellet with granule remnants (GRs). Granule remnants were re-suspended in 1 ml of serum-free medium.

Cell culture and treatments

In this study, we used two human hepatocellular carcinoma cell lines, HuH-6 and HA22T/VGH, with different characteristics of differentiation and biological behavior. HA22T/VGH is a poorly-differentiated hepatocellular carcinoma cell line which contains HBV integrants and HuH-6 is a well-differentiated hepatoblastoma-derived cell line. In addition, the different cell lines also have different genetic defects. For example, a major oncogenic alteration of HuH-6 cells is their aberrant expression of β-catenin (De La Coste *et al.*, 1998; Carruba *et al.*, 1999). The cell lines had a narrow range of passage number and were maintained in MEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS in 5% humidified CO_2 at 37°C. The HCC cells were plated at 1×10^5 in a 24-well plate in MEM medium with 10% FCS and left overnight to adhere. After removing the medium the cells were incubated for 24 h with or without 1 ml of total mast cell releasate, granule remnants and granule remnant-free supernatants. In all the experiments studying the effect of histamine, the cells were cultured in a medium containing 1% FCS. In the combination experiments of ranitidine or terfenadine with histamine the latter was added 10 min after the two receptor antagonists. The cells were rinsed twice with PBS buffer and then lysated with ice-cold RIPA-buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5%

sodium deoxycholate (DOC), 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml each apoprotinin, pepstatin, leupeptin). The cell lysates were kept for 30 min on ice and insoluble material was then removed by centrifugation at 10,000 $\times$ g for 20 min at 4°C. The protein concentration of the supernatant was determined using a standard Bio-Rad protein assay (Bio-Rad Laboratories, CA).

Western blot analysis

Identical amounts of protein (25 μg) were resolved onto 12% SDS-PAGE for COX-2 analysis, and onto 10% SDS-PAGE for β-actin, caspase 3, PARP, survivin, and β-catenin analyses. SDS-PAGE gels were electro-transferred to nitrocellulose (Amersham Pharmacia Biotech., Milan, Italy), and probed with primary antibodies. Immunolabeling was detected by enhanced chemiluminescence (ECL plus[TM] Amersham Pharmacia Biotech., Milan, Italy). Intensities of protein bands were quantified by computerized densitometry using Molecular Analysis Software (Bio-Rad Laboratories).

RT-PCR analysis

Total RNA was isolated from HuH-6 and HA22T/VGH (2 $\times$ 10^5) cells using Tryzol reagent (Invitrogen, Carlsbad, CA) as recommended by the supplier. 3 μg of total RNA was submitted to reverse transcription to generate cDNA by using the Advantage RT-PCR kit (Clontech, CA). To analyze human COX-2, survivin, H_1 and H_2 receptors mRNA expression levels, semi-quantitative PCR was performed using specific 5' and 3' primers which generated specific bands (Table 1). Amplification of cDNA was performed with the following number of cycles: 40 for H_1 and H_2 receptors in HuH-6 and HA22T/VGH cells, and for COX-2 in HuH-6 cells; 35 cycles for survivin in HuH-6 and HA22T/VGH cells, and for COX-2 in HA22T/VGH cells; 28 cycles for β-actin in both cell lines. Aliquots of the PCR products (10 μl) were fractionated by electrophoresis using 1.6% agarose gel containing ethidium bromide, visualized under UV light and photographed.

Evaluation of cell growth by MTS assay

Cells (5 $\times$ 10^3/well) in complete medium were distributed into each well of 96-well microtiter plates and incubated overnight. The medium was then removed and either 100 μl total medium, granule remnant-free supernatants and granule remnants, or 100 μl histamine at different concentrations in serum-free medium were added. In the experiments with histamine receptor antagonists cells were pre-incubated for 10 min with antagonists before histamine addition. After 24 h, 15 μl of a commercial solution (obtained from Promega Corporation, Madison, WI) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate were added to each well. The plates were incubated for 1 h in a humidified atmosphere at 37°C in 5% CO_2. The bioreduction of the MTS dye was assessed by measuring the absorbance of each well at 490 nm. Cytotoxicity was expressed as a percentage of the absorbance measured in the control cells. Values were expressed as means $\pm$ SE of three separate experiments, each performed in triplicate.

Cell proliferation assay

Cell proliferation was determined by estimating the amount of bromodeoxyuridine (BrdU) incorporation into DNA by a colorimetric immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 2.5 $\times$ 10^3 cells were cultured in 96-well plates in the presence of histamine at different concentrations for 8 h. BrdU (10 μM final concentration) was added, and the cells were re-incubated for an additional 16 h. The cells were fixed and incubated with anti-BrdU-POD according to the manufacturer's instructions. The color was developed by the addition of tetramethylbenzidine substrate and measured at 490 nm. The color intensity and absorbance values directly correlate to the amount of BrdU incorporated into DNA. The results were expressed as the percentage inhibition of BrdU incorporation over control. Values were expressed as means $\pm$ SE of three separate experiments, each performed in triplicate.

Table 1. Specific primers for RT-PCR.

Gene	Sense primer	Antisense primer	Annealing (^{o}C)	Size (bp)
β-actin	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	56	175
COX-2	GAGAAAACTGCTCAACACCG	GCATACTCTGTTGTGTTCCC	60	745
Survivin	GCATGGGTGCCCCGACGTTG	TCCGGCCAGAGGCCTCAA	60	447
H_1	AAGTCACCATCCCAAACCCCCAAG	TCAGGCCCTGCTCATCTGTCTTGA	62	195
H_2	AGGAACGAGACCAGCAAGGGCAAT	GTGGCTGCCTTCCAGGAGCTAAT	62	197

Statistical analysis

One-way ANOVA was used to determine differences between groups. When differences were found to exist, ANOVA analysis was followed by a two-tailed Student's test and a *P* value < 0.05 was considered significant.

Results

Effects of mast cell mediators on HCC cell growth

We treated HuH-6 and HA22T/VGH cells for 24 h with conditioned medium derived from mast cells cultured *in vitro* as described in Materials and Methods. The treatment of both cell lines with total "mast cell releasate" resulted in a significant decrease in cell viability only in HuH-6 cells, as assessed by MTS assay (Figure 1A). When we exposed the two cell lines to granule remnant-free supernatant (GR-FS) no significant alterations in cell growth were observed in either of the cell lines.

Treatment with granule remnants (GRs) resulted in a significant inhibition of HuH-6 cell growth and a slight but significant increase in HA22T/VGH cell growth (Figure 1A).

Since histamine is one of the most abundant mediators present in the spontaneously-released mast cell granules and it has been shown to exert pro- or anti-tumor activity (Reynolds *et al.*, 1996; Lazar-Molnar *et al.*, 2002), we explored its possible role in the growth of the two cell lines. The effects of histamine on HCC cells were first evaluated by MTS assay and, as in the case of GRs treatment, histamine showed opposite effects on the two cell lines (Figure 1B). In fact, histamine inhibited the cell growth of HuH-6 cells in a dose-dependent manner (from 0.01 to 1 μM), whereas it weakly promoted cell growth in HA22T/VGH cells (Figure 1B). In Figure 1B are also shown the results obtained in human colon cancer Caco-2 cells treated with histamine, used as a control. The exogenous histamine stimulated cell growth in Caco-2 cells at 1 μM, as shown
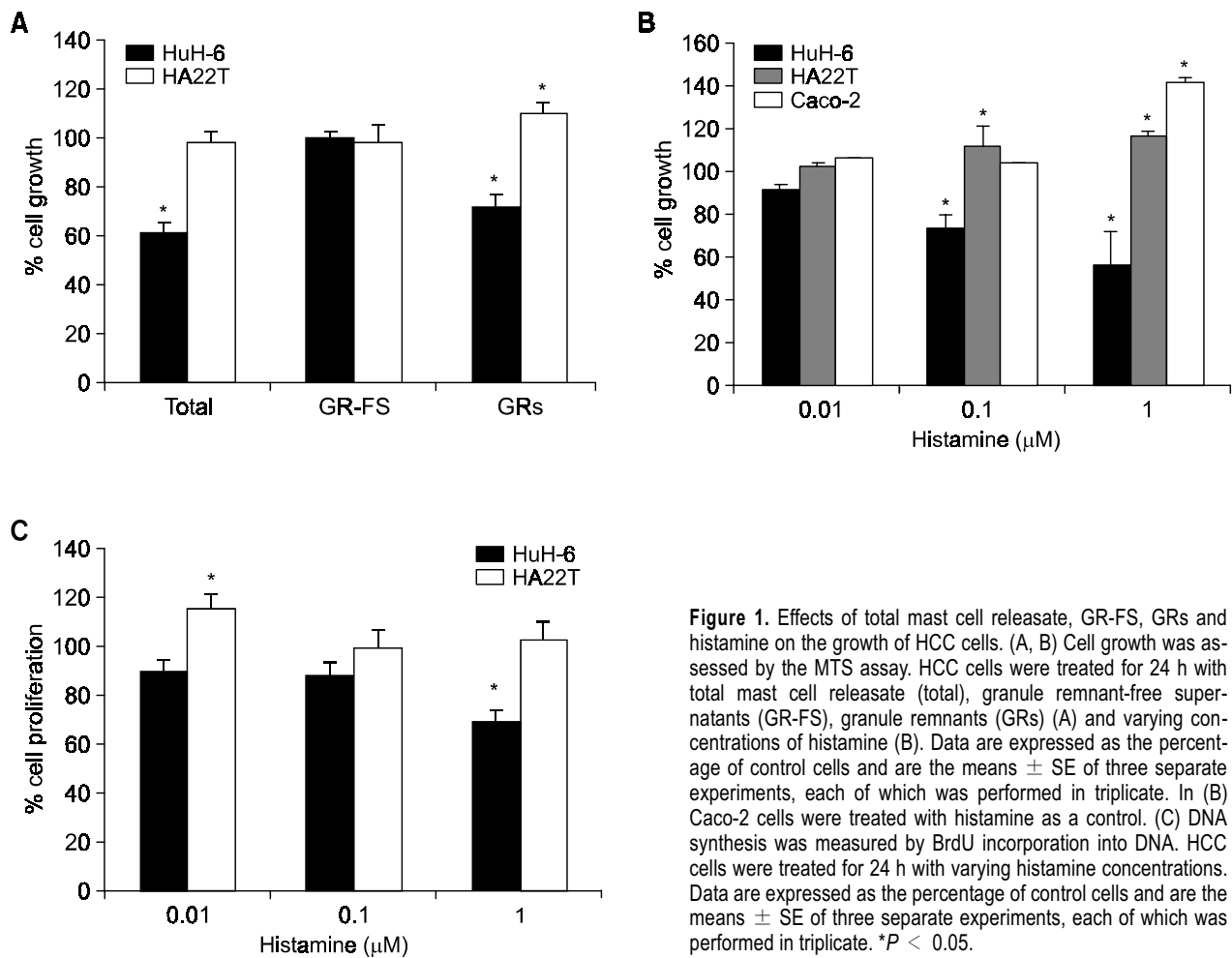

Figure 1. Effects of total mast cell releasate, GR-FS, GRs and histamine on the growth of HCC cells. (A, B) Cell growth was assessed by the MTS assay. HCC cells were treated for 24 h with total mast cell releasate (total), granule remnant-free supernatants (GR-FS), granule remnants (GRs) (A) and varying concentrations of histamine (B). Data are expressed as the percentage of control cells and are the means ± SE of three separate experiments, each of which was performed in triplicate. In (B) Caco-2 cells were treated with histamine as a control. (C) DNA synthesis was measured by BrdU incorporation into DNA. HCC cells were treated for 24 h with varying histamine concentrations. Data are expressed as the percentage of control cells and are the means ± SE of three separate experiments, each of which was performed in triplicate. *$P < 0.05$.

by Cianchi *et al.* (2005). To explore whether the growth inhibitory effect was also associated with a change in the proliferation rate, DNA synthesis was quantified by BrdU uptake in HCC cells. As shown in Figure 1C, histamine inhibited DNA synthesis in HuH-6 cells, whereas a weak stimulation was observed in HA22T/VGH cells.

All these results suggest that mast cell mediators may affect HCC cell growth and histamine in particular may have a dual role in cell growth, increasing the proliferation of the poorly-differentiated HA22T/VGH cells and inhibiting the proliferation of the well-differentiated HuH-6 cells.

Expression of histamine H_1 and H_2 receptors and the effects of histamine receptor antagonists in HCC cells

Since histamine can decrease cell proliferation through the H_1 receptor, whereas it enhances cell proliferation through the H_2 receptor (Wang *et al.*,

1997; Liu *et al.*, 2003), we investigated for the presence of H_1 and H_2 receptors and their association with cell growth in HCC cells. RT-PCR analyses showed that both the HCC cell lines expressed H_1 and H_2 receptor mRNA, suggesting that histamine may effectively trigger HCC cell response through its receptors. The Caco-2 human colon cancer cell line was used as a positive control for H_1 and H_2 receptor mRNA expression (Figure 2A) (Cianchi *et al.*, 2005).

The selective H_1 and H_2 receptor antagonists, terfenadine and ranitidine respectively, were thus used alone or in combination with histamine in MTS experiments. Terfenadine (1 μM) and ranitidine (1 μM) when used alone did not affect the growth of HCC cells (Figure 2B-C). Terfenadine in combination with histamine (1 μM) prevented histamine-induced inhibition of cell growth in HuH-6 cells (Figure 2B), whereas it did not have any suppressive effect in HA22T/VGH cells (Figure 2C). On the contrary,
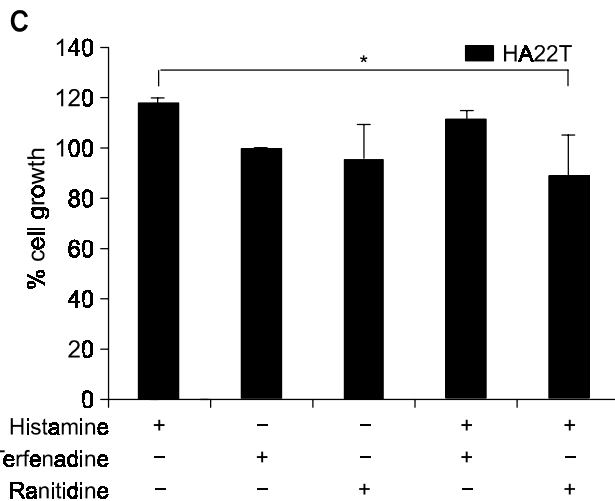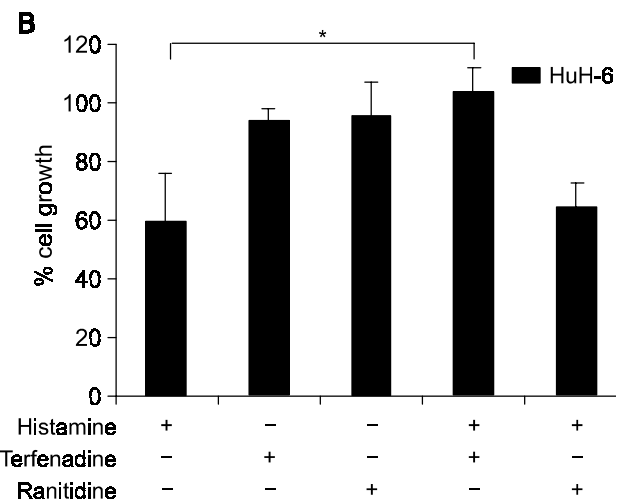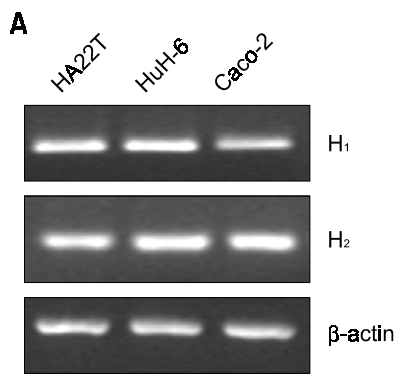

Figure 2. Expression of histamine receptors and the effects of histamine receptor antagonists on HCC cells. (A) mRNA expression for H_1 and H_2 receptors were detected in HCC cell lines by RT-PCR analysis. As a control, the expressions of both receptors in human colon cancer Caco-2 cells were also analyzed. (B, C) The effects of terfenadine (1 μM), as well as ranitidine (1 μM) alone or in combination with histamine (1 μM) for 24 h on HuH-6 (B) and on HA22T/VGH (C) cell growth were measured by MTS assay. Data are expressed as the percentage of control cells and are the means ± SE of three separate experiments, each of which was performed in triplicate. *$P < 0.05$.

ranitidine in combination with histamine prevented histamine-induced cell growth in HA22T/VGH cells (Figure 2C) but did not revert histamine-induced inhibition in HuH-6 cells (Figure 2B). These results suggest that histamine triggered the inhibition of cell growth in HuH-6 cells, possibly through the H_1 receptor, while it increased cell growth in HA22T/VGH cells, possibly through the H_2 receptor.

Histamine affects β-catenin expression in HCC cells

The molecular mechanisms by which histamine controls cell proliferation are unknown. However, it was recently shown that the canonical β-catenin pathway acts down-stream of the histamine receptor H_1 in a variety of cell types, decreasing β-catenin content (Diks *et al.*, 2003).

We therefore investigated whether treatment of HCC cells with GR-FS, GRs and histamine may have an effect on β-catenin protein expression

levels. Western blot analysis showed that GRs and histamine decreased the level of β-catenin in HuH-6 cells, whereas they increased β-catenin expression in HA22T/VGH cells (Figure 3), suggesting that β-catenin protein might be a molecular target for mast cell mediators in HCC cells.

Histamine affects survivin protein expression in HCC cells

Recent evidence indicates that the expression of the inhibitor of apoptosis protein (IAP) survivin is a target of β-catenin/Tcf signaling (Zhang *et al.*, 2001) and that it inhibits apoptosis by binding to active caspase-3 and caspase-7 (LaCasse *et al.*, 1998). Moreover, survivin is also required for cell division (Li *et al.*, 1998), and in clinical HCC samples in particular its expression has been shown to be strongly correlated with the proliferation index (Ito *et al.*, 2000). Therefore, experiments were carried out
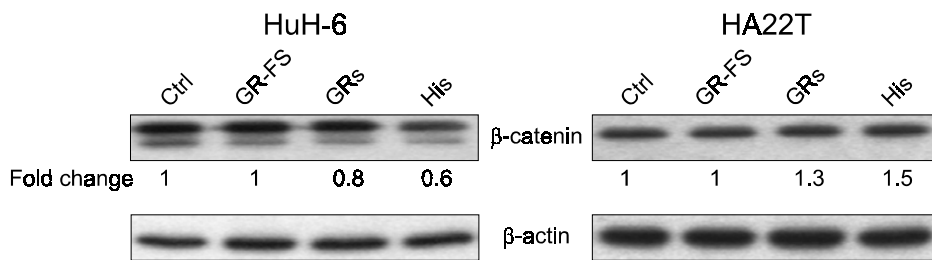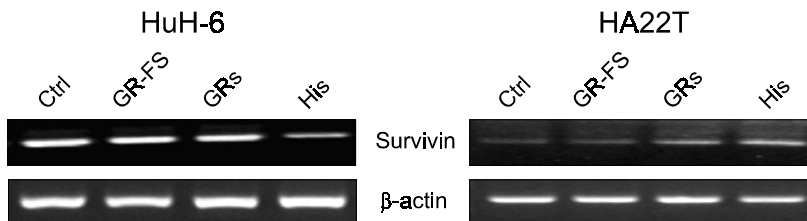

Figure 3. Effects of GR-FS, GRs supernatants and histamine on β-catenin expression. Cells were untreated (Ctrl) or treated for 24 h with GR-FS, GRs and histamine (0.5 μM) for 24 h and then β-catenin expression was analyzed by western blot. 25 μg of protein were loaded. The intensity of the resulting bands was evaluated by densitometry analysis and the β-catenin/β-actin ratio was reported. The numbers beneath the blots represent the ratio with vehicle-treated control samples (Ctrl) arbitrarily set at 1.0. Representative immunoblots of results obtained from three independent experiments are shown.
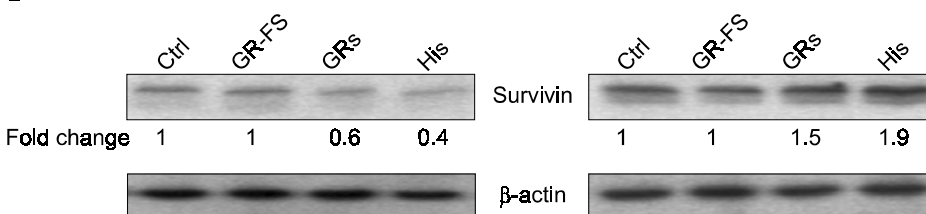

Figure 4. Effects of GR-FS, GRs and histamine on survivin expression. Cells were untreated (Ctrl) or treated for 24 h with GR-FS, GRs and histamine (0.5 μM) and then β-actin and survivin mRNAs and protein expression levels were analyzed by RT-PCR (A) and western blot (B), respectively. In (B) 25 μg of protein were loaded. The intensity of the resulting bands was evaluated by densitometry analysis and the survivin/β-actin ratio was reported. The numbers beneath the blots represent the ratio with vehicle-treated control samples (Ctrl) arbitrarily set at 1.0. Representative gels and immunoblots of results obtained from three independent experiments are shown.

to investigate the potential effects of mast cell mediators on the expression of survivin in HCC cells. As shown in Figure 4, after exposure of HuH-6 cells to GRs and histamine for 24 h, an inhibition of both survivin mRNA and protein expression levels was observed, whereas in HA22T/VGH cells survivin expression was enhanced. These results suggest that GRs and histamine might affect the survival of HuH-6 cells presumably also by survivin down-regulation, while in HA22T/VGH cells the up-regulation of survivin expression could be linked to enhanced cell growth.

Histamine induces the activation of caspase-3 in HuH-6 cells

Survivin and other inhibitors of apoptosis protein (IAPs) have been shown to inhibit apoptosis by binding to active caspases (LaCasse et al., 1998). Therefore, it is reasonable to hypothesize that as survivin levels decrease the activity of caspases could increase. Therefore, we evaluated capsase-3 activation in the HCC cell lines after treatment with GR-FS, GRs and histamine. Cells were treated for 24 h and then whole cellular extracts were analyzed by western blot. In Figure 5, we showed that the presence of the 20 kDa and 17 kDa fragments generated by the cleavage of pro-caspase-3 was enhanced in HuH-6 cells as a consequence of the treatment with GRs and histamine, whereas in HA22T/VGH cells no signs of caspase-3 activation were visible. Indeed, there was a post-treatment decrease in the 20 kDa fragment of treated cells when compared with controls, in which the 20 kDa fragment was observed due to spontaneous apoptosis (Figure 5). The histamine-induced caspase-3 activation is also suggested by the presence of the cleaved form of the caspase-3 target PARP (Figure 7A lane 2). Caspase activation is a hallmark

of the apoptosis process, therefore these results suggested that the reduction in cell growth in HuH-6 cells following GRs and histamine treatment is probably linked to the induction of the apoptosis cascade, through the activation of caspase-3.

The expression of COX-2 is affected by GR supernatants and histamine in HCC cells

Several lines of evidence indicate that COX-2 is important for HCC cell growth (Hu et al., 2003; Fodera et al., 2004; Sung et al., 2004; Wu, 2006). Moreover, according to Cianchi et al. (2005) histamine exerts a proliferative effect in Caco-2 colon cancer cells through the activation of H_2/H_4 receptors, increasing COX-2 expression and in turn PGE_2 production. In addition, COX-2 is a β-catenin gene target (Mei et al., 1999), and our results showed that histamine affected β-catenin expression. Therefore, we investigated whether mast cell mediators affected the expression of COX-2 mRNA and protein in HCC cell lines using RT-PCR and Western blot analyses, respectively. HuH-6 and HA22T/VGH cells were incubated with GRs and histamine for 24 h and then the expressions of COX-2 mRNA and protein were analyzed. The expressions of both COX-2 mRNA (Figure 6A) and protein (Figure 6B) were reduced in HuH-6 cells treated with histamine. On the contrary, in HA22T/VGH cells GRs and histamine treatment resulted in an increase in COX-2 mRNA (Figure 6A), whereas with histamine a weak increase in protein expression was obtained (Figure 6B).

Overall, these data suggested that mast cell mediators might have an inhibitory effect on HuH-6 cell growth, possibly through the reduction of COX-2 expression, whereas they may stimulate HA22T/VGH cell growth by increasing COX-2 protein expression.
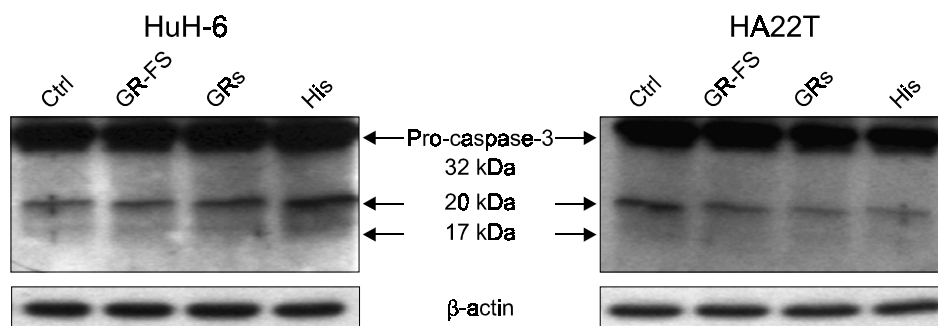

Figure 5. Effects of GF-FS, GR supernatants and histamine on caspase-3 activation. Cells were untreated (Ctrl) or treated for 24 h with GR-FS, GRs and histamine (0.5 μM). Whole cellular extracts were analyzed by western blot for β-actin and caspase-3 protein expression. 25 μg of protein were loaded. Fragments of 20 and 17 kDa obtained by cleavage of pro-caspase-3 are shown. Representative immunoblots of results obtained from two independent experiments are shown.
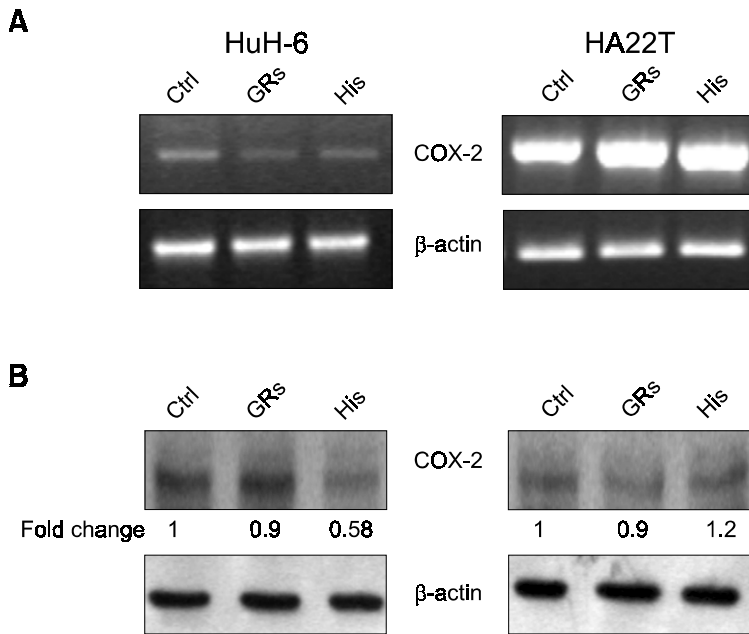
A

B

Fold change   1   0.9   0.58   1   0.9   1.2

Figure 6. Effects of GRs and histamine on COX-2 expression. Cells were untreated (Ctrl) or treated for 24 h with GRs and histamine (0.5 μM). Post-treatment β-actin and COX-2 mRNAs (A) and protein (B) expression levels were analyzed by RT-PCR and western blot, respectively. In (B) 25 μg of protein were loaded. The intensity of the resulting bands was evaluated by densitometry analysis and the COX-2/β-actin ratio was reported. The numbers beneath the blots represent the ratio with vehicle-treated control samples (Ctrl) arbitrarily set at 1.0. Representative gels and immunoblots of results obtained from two independent experiments are shown.
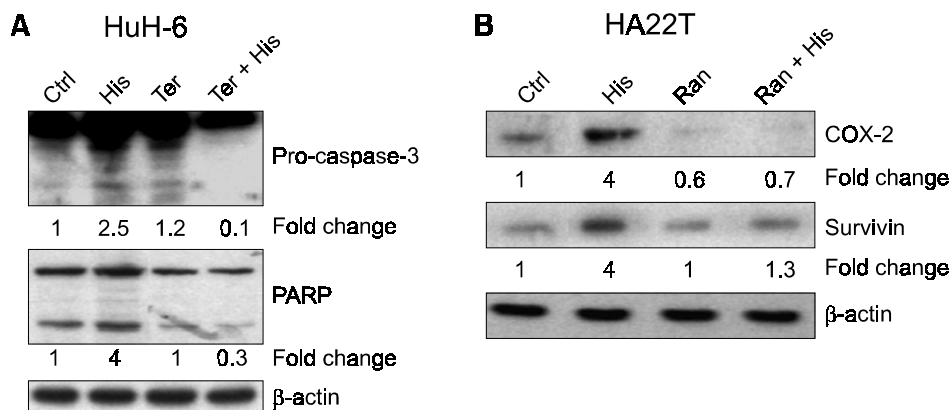
A   HuH-6   **B**   HA22T

Figure 7. Effects of H_1 and H_2 receptor antagonists on caspase-3 activation, PARP cleavage, COX-2 and survivin expression. Cells were untreated (Ctrl) or treated for 24 h with histamine (0.5 μM) in the presence or absence of terfenadine (1 μM) in HuH-6 cells and of ranitidine (1 μM) in HA22T/VGH cells. Protein expression levels were analyzed by western blot. 25 μg of protein were loaded. (A) The H_1 receptor antagonist terfenadine can block the histamine-induced caspase-3 activation and in turn PARP cleavage in HuH-6 cells. (B) The H_2 receptor antagonist ranitidine reversed the over-expression of COX-2 and survivin induced by histamine in HA22T/VGH cells. The intensity of the resulting bands was evaluated by densitometry analysis and the target/β-actin ratio was reported. The numbers beneath the blots represent the ratio with vehicle-treated control samples (Ctrl) arbitrarily set at 1.0. The numbers beneath the blots of pro-caspase 3 and PARP refer to the fragments quantification. Representative immunoblots of results obtained from three independent experiments are shown.

The selective receptor antagonists, terfenadine and ranitidine, can block the effects of histamine

To confirm the role of histamine H_1 and H_2 receptors in HCC cell growth we treated the cells with histamine in the presence of terfenadine (1 μM) and of ranitidine (1 μM). As shown in Figure 7A terfenadine blocked histamine-induced caspase-3 activation and in turn PARP cleavage in HuH-6 cells, whereas in HA22T/VGH cells ranitidine prevented histamine-induced COX-2 and survivin over-expression (Figure 7B).

Discussion

To clarify the role of mast cells in the liver malignancies we investigated in the present study the involvement of mast cell mediators in the cell growth of two human hepatocellular carcinoma cell lines, HuH-6 and HA22T/VGH. Our results showed that, total mast cell releasate, exocytosed granules (granule remnants), and histamine reduced the cell viability and proliferation of HuH-6 cells. On the contrary, in HA22T/VGH cells granule remnants and histamine induced a slight increase in cell growth. It has been reported that histamine may have a dual role in the proliferation of melanoma cells, depending on its local concentration (Lazar-Molnar *et al.*, 2002) or on the type and balance of histamine receptors in the cells (Falus *et al.*, 2001). We found that HCC cell lines possessed both H_1 and H_2 histamine receptors, but responded differently to histamine. Therefore, it is reasonable to hypothesize a different role for histamine receptors in the two cell lines. Accordingly, our experiments with H_1 and H_2 antagonists showed a different linkage between the two receptors and cell growth.

The mechanism by which histamine leads to cell proliferation or apoptosis is unknown. However, it has recently been shown that at least the H_1 receptor is coupled with the canonical β-catenin pathway (Diks *et al.*, 2003), and once activated this pathway regulates the expression of different target genes which are involved in the control of cell proliferation and apoptosis, including COX-2 (Mei *et al.*, 1999) and survivin (Zhang *et al.*, 2001). We reported that HA22T/VGH cells treated with histamine accumulated β-catenin and this was also linked to the up-regulation of COX-2 and survivin. This might result in an increased cell survival, probably through the H_2 histamine receptor, in accordance with the results observed in the presence of the specific receptor antagonist ranitidine. On the contrary, in HuH-6 cells the level of β-catenin protein was decreased by histamine. This was linked to reduced COX-2 and survivin expression and also to the inhibition of cell survival/proliferation, probably through the H_1 histamine receptor, as suggested by the results observed in the presence of the specific receptor antagonist terfenadine.

Exogenous histamine has been reported to be involved in human colon cancer cell proliferation through the up-regulation of COX-2 protein expression (Cianchi *et al.*, 2005). We showed that histamine induces an up-regulation of COX-2 mRNA and protein expression in HA22T/VGH cells, whereas in HuH-6 cells it inhibits COX-2 expression. In HCC cells COX-2 over-expression and activity have been linked to cell survival and proliferation (Leng *et al.*, 2003; Fodera *et al.*, 2004; Lampiasi *et al.*, 2006). In agreement, we found that an increase in cell survival was linked to COX-2 expression in HA22T/VGH cells, while the opposite was observed in HuH-6 cells.

Apoptotic cell death may offer an alternative explanation for the reduced cell growth observed in HuH-6 cells. Survivin is a member of the IAP family and is able to bind caspases, thus inhibiting their activity (LaCasse *et al.*, 1998). In our experiments we found that in HuH-6 cells GRs and histamine reduced survivin expression levels and also induced caspase-3 activation, suggesting a possible increase in apoptosis. Instead, in HA22T/VGH cells the increase in survivin expression could be related to cell proliferation. These results are in accordance with the observation that survivin is negatively regulated during apoptosis (Ikeguchi *et al.*, 2002), whereas it is positively correlated with HCC cell growth (Ito *et al.*, 2000).

In conclusion, the results presented here underline that mast cell mediators and histamine may affect the growth of HCC cells. Furthermore, they support the possible use of histamine and histamine receptor agonists/antagonists in the treatment of this cancer. Since hepatocellular carcinoma is one of the sixth major causes of death worldwide and current drug treatment is unsuccessful, the development of "new drugs" is of paramount importance.

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