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Enclosures
Histamine Inhibits Cell Proliferation and Modulates the Expression of Bcl-2 Family Proteins via the H2 Receptor in Human Pancreatic Cancer Cells

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Abstract. In this study, the mechanisms involved in the inhibitory effect of histamine (HA) on PANC-1 cell proliferation were investigated. The action of HA on cell growth was evaluated using the determination of the cell doubling time from experimental growth curves and cell cycle analysis using a flow cytometer. The expression of proteins related to cell death and proliferation (PCNA, p53, c-Fos and Bcl-2 family proteins) was studied using Western blot, immunocytochemistry and flow cytometric analysis. The results indicated that HA produced an accumulation of PANC-1 cells in G0/G1 phase and increased the doubling time via H2 histamine receptor (H2R) stimulation. Expression of p53, c-Fos and Bcl-2 were not modulated by HA. However, HA decreased PCNA and Bax expression, while it increased the Bcl-x level. In summary, the antiproliferative effect exerted by HA was associated with a G0/G1 phase arrest and a modulation of the Bcl-2 family proteins.

Pancreatic adenocarcinoma is a leading cause of cancer death worldwide. This lethal disease is characterized by an extremely poor prognosis because of its aggressive nature and lack of response to conventional therapy. On this basis, new therapeutic modalities are currently being evaluated for pancreatic cancer.

As an approach to the understanding of the molecular biology of this neoplasm, the human pancreatic carcinoma cell line PANC-1 was employed to investigate the in vitro effects of HA on cell growth. The PANC-1 cell line was derived from a human pancreatic carcinoma of ductal origin. This poorly differentiated epithelioid cell line, which harbours mutated \( p53 \), was originally established by Lieber (1).

HA, a biogenic amine widely distributed throughout the body, is involved in the regulation of diverse physiological and pathological processes and exerts its actions through the activation of four classes of membrane G protein-coupled receptors (H1R, H2R, H3R and H4R). HA receptors have also been characterized in a variety of cell lines and human neoplasias. Several studies have shown that HA may regulate cell growth via these receptors (2).

We previously reported that PANC-1 cells overexpress H1R and H2R. HA concentrations higher than 1 \( \mu \)M activate the adenylate cyclase pathway by H2R stimulation, increase 3',5'-cyclic adenosine monophosphate (cAMP) production and exert an inhibitory effect on cell proliferation (3). Apoptosis is not significantly induced by HA in this cell line. However, a partial cell differentiation is associated with the inhibitory action of HA (4).

Bax, Bcl-2 and Bcl-x are members of a large family of intracellular proteins described as important positive and negative regulators of apoptosis. It is well known that the antiapoptotic Bcl-2 members bind the proapoptotic proteins to counteract one another's function, suggesting that their relative concentration may act as a rheostat for cell death programme (5). Moreover, there is general agreement that Bax is needed to induce apoptosis (6, 7). Recent reports confirmed that apoptosis of cancer cells induced by several chemotherapy agents is dependent on Bax (8) and that epithelial cancer cells lacking Bax are resistant to apoptosis (9).

The aim of this work was to investigate the mechanism involved in the inhibitory, not the apoptotic, effect of HA on PANC-1 cell growth by the analysis of cell cycle and the study of proteins related to cell death and proliferation.
Materials and Methods

Materials. Histamine dihydrochloride, mouse monoclonal antibody anti-β-actin (clone AC-15), mouse monoclonal anti-rabbit IgG (Gamma) peroxidase conjugate clone RG-96, rabbit anti-mouse IgG (whole molecule) peroxidase conjugate, goat anti-rabbit IgG (whole molecule) and goat anti-mouse both conjugated to fluorescein isothiocyanate (FICT) were purchased from Sigma Chemical Co (St. Louis, MO, USA). The H2 agonist dimaprit dihydrochloride (Dim) was from Tocris Cookson, Inc (MO, USA). Forskolin (Fk) was from Calbiochem (Merck Biosciences, USA). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco, Invitrogen (CA, USA). Rabbit polyclonal antibodies against human Bax (N-20), Bcl-2 (A21), Bcl-xL (S-18) and c-Fos (4-G) were from Santa Cruz Biotechnology, Inc (CA, USA). Mouse monoclonal antibodies against p53 (DO-1 clone) and the proliferating cellular nuclear antigen (PCNA) were from Santa Cruz Biotechnology (CA, USA) and DakoCytomation (CA, USA) respectively. The Enhanced Chemiluminescence System (ECL) was from Amersham Biosciences Argentina S.A.

Cell culture. PANC-1 (ATCC CRL 1649) cells were grown in monolayer in RPMI medium supplemented with 10% fetal bovine serum (FBS), 0.3 g/l L-glutamine and 40 mg/l gentamycin, at 37°C in a 5% CO2 humidified atmosphere.

Cell population doubling time. PANC-1 cells were seeded on plates at a density of 1x105, starved for 24 h and then incubated with or without 10 μM HA or 10 μM Dim in RPMI medium containing 10% FBS. After 24, 48 and 72 h cells were harvested and trypsinised. Viable cells were counted using a hemocytometer. All experiments were performed in the logarithmic phase of cell growth. Triplicate plates were analyzed for each treatment and time. Experimental curves were adjusted to the equation: N = N0 cells x t, using the GraphPad Prism 4.0 software (GraphPad Software Inc, Philadelphia, USA). Cell Doubling time (T) was determined as: T = ln 2/ka.

Cell cycle analysis. After synchronization, cells were incubated as previously described. At 24, 48 and 72 h they were fixed with 1 ml ice-cold 70% ethanol, centrifuged and resuspended in 100 μl Dnase-free RNase A (0.2 mg/ml). Cells were stained with propidium iodide (50 μg/ml), stored at 4°C in the dark and analyzed by flow cytometry on a FACSCalibur instrument (Becton-Dickinson, CA, USA) within 2 h. Red fluorescence was measured using a fluorescence detector 2 (FL2) on the X-axis. Cell cycle phase distribution, i.e., the percentage of G0/G1, S and G2/M nuclei of the analyzed cell population, was determined by applying Cypher 1.0.2 cell cycle analysis software (Cardiff University, Cardiff, UK) on the DNA histograms.

Western blotting. Synchronized cells were cultured with or without 10 μM HA, 10 μM Dim or 10 μM Fk. Bcl-2 family proteins, PCNA and p53 expression were studied at 24 and 48 h using Western blot while c-Fos was studied at 2, 4, 12 and 24 h. Cells were scraped into lysis buffer (0.1 mM sodium fluoride, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1mM ethylene glycol tetra acetate acid (EGTA), 1 mM sodium vanadate, 0.02 mg/ml leupeptin and 100 mM Tris (pH 8.8) at 4°C. After centrifugation, supernatants were boiled for 3 min in reducing conditions. Protein concentration was determined using the Bradford method (10). Fifty μg of protein were separated on a 12% sodium dodecylsulfate polyacrylamide gel by electrophoresis and transblotted onto nitrocellulose membranes. Membranes were blocked in Tris-buffered saline/Tween (25 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% (w/v) nonfat dried milk. Blots were probed overnight at 4°C with the rabbit antibodies (1: 200) against Fos, Bcl-2, Bcl-x and Bax or with the mouse antibodies anti PCNA (1: 500) and anti p53 (1: 200). Immunoreactivity was developed using anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugate antibody, as appropriate, and detected by the enhanced chemiluminescence reagents following the manufacturer’s instructions. Blots were re-probed with mouse antibody against β-actin (1:1000) to control for equal protein loading of the gel. The relative expression level of proteins was determined by comparing signal intensity to β-actin bands and was quantitated by densitometry employing the image J 1.32 software (NIH, USA).

Flow cytometric analysis. PANC-1 cells were cultured as previously described for Bcl-2, Bax, PCNA and c-Fos detection. The nonspecific background staining was controlled with a rabbit or mouse IgG isotype control as appropriate. Cells were fixed in 4% formaldehyde in phosphate buffered-saline (PBS), centrifuged and permeabilized with 0.1% saponin in PBS. After centrifugation, the first appropriate antibody (1:100) was added for 60 minutes at room temperature. Cells were washed with 0.1% saponin, treated with the anti-mouse or anti-rabbit FITC conjugate antibody (1:200) and incubated in darkness. They were then washed and resuspended in 2% formaldehyde in PBS, ready to be analyzed by flow cytometry on a FACSCalibur instrument using WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

Immunocytochemistry. Cells were cultured on glass slides for 24 and 48 h with or without 10 μM HA or Dim. They were then fixed with 4% formaldehyde in PBS, permeabilized with 0.25% Triton and incubated overnight at 4°C with a rabbit polyclonal antibody against Bcl-2 or Bax followed by the appropriate FITC-labelled antibody for one hour at room temperature in the darkness. The nonspecific background staining was controlled as previously indicated. Stained cells were examined with a fluorescence microscope (Karl Zeiss, Germany) bearing a digital camera.

Statistical analysis. Statistical analysis performed is indicated in the legends. Data were analysed by One-way or Two-way ANOVA and Bonferroni or Dunnett post-test, using the GraphPad Prism Version 4.00 software (GraphPad Software Inc, Philadelphia, USA). P values less than 0.05 were considered statistically significant.

Results.

HA enlarges the cell doubling time and induces G0/G1 cell cycle arrest. To investigate the antiproliferative effect induced by HA via H2R on PANC-1 clonogenic growth (3), we analysed the population doubling time from experimental growth curves when cells were cultured in monolayers with or without HA or Dim. As shown in Figure 1, the cell number at 72 h in cultures exposed to HA or Dim was
significantly lower than in the control. This would appear to be a time-dependent effect since there were no significant differences up to 24 h. Doubling Time was determined from the experimental growth curves as detailed in Materials and Methods. An important increase in the T-value was observed when cells were cultured in the presence of 10 μM HA or Dim (1.6-fold higher than untreated cells) indicating that HA and Dim slowed down the cell cycle (Table I).

In order to determine whether HA might induce cell cycle arrest via H2R, we carried out the cell cycle analysis of PANC-1 cells cultured with or without 10 μM HA or Dim. Results revealed that HA changed the cell cycle distribution. At 48h and 72 h there was an increased accumulation of G0/G1 phase cells for HA-treated cultures compared to control cultures (Figure 2A). Similar results were obtained when cells were grown in the presence of 10 μM Dim (Figure 2B), indicating a retardation of cell cycle progression coincident with the larger T-value mentioned above.

Effects of HA on the expression of proteins related to cell death and proliferation. Seeking HA-induced factors potentially influencing the balance between cell survival and cell death, we evaluated the expression pattern of PCNA, c-Fos, p53 and the Bcl-2 family proteins in PANC-1 cell cultures. In view of the known stimulatory action of H2R on adenylate cyclase/cAMP, we examined whether modulation of these protein levels was via the cAMP signaling pathway. For this purpose, cells were exposed to HA, the H2 agonist Dim, or the direct activator of adenylate cyclase, Fk.

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<td>Control</td>
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<td>10 μM HA</td>
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<tr>
<td>10 μM Dim</td>
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* p<0.05 vs. Control, Two-way ANOVA and Bonferroni post-test.
At 48 h, a significant decrease in PCNA expression, a marker of cell proliferative activity, was observed using Western blot (Figure 3A) and flow cytometry (Figure 3B). The PCNA level was down-modulated by HA, Dim and Fk concomitantly with a reduction in the rate of population growth in cultures treated with HA or Dim as shown in Figure 1.

At 24 h, the expression of mutated p53 protein was not modified by treatment (Figure 4). In addition, no change in c-Fos level was detected by Western blot when cells were treated with HA for 2, 4, 12 and 24 h or when cells were cultured with HA, Dim or Fk for 4 h (Figure 5). Similar results were obtained at 2, 12 and 24 h and were corroborated by flow cytometric analysis (data not shown).

At 24 h, Bcl-2 could not be detected by the assayed
techniques in the control, nor the treated cells. Immunocytochemical studies displayed no fluorescence staining for Bcl-2 when treated and control cells were irradiated with the excitation light (Figures 6A-1 and 6A-3). Observation of the same fields without excitation light confirmed that cells were present on the slides (Figures 6A-2 and 6A-4). These data were corroborated by flow cytometric analysis (Figure 6, lower panel). Similar results were obtained at 48 h.

Bax protein expression was detected by immunocytochemistry in both treated and untreated cultures (Figure 6B). However, a lower level of Bax protein expression in HA-exposed cells compared to control cells was evidenced by flow cytometric analysis (Figure 6, lower panel). These results were also observed by Western blotting (Figure 7). In addition, two isoforms of Bcl-x protein, the antiapoptotic Bcl-xL and the proapoptotic Bcl-xS were also visualized using the Western blot technique (Figure 7). The expression of both isoforms was augmented significantly in cells receiving HA, Dim or Fk at 24 h, the enhancement of Bcl-xL being larger than Bcl-xS for each treatment. Similar data were acquired at 48 h (data not shown).

**Discussion**

HA has been proposed as a potential modulator of exocrine pancreatic function acting through H1R and H2R in the normal pancreas (11). However, the role of HA and
these receptors in pancreatic cancer have not yet been elucidated.

There is a large amount of data available regarding the effects of HA on normal and neoplastic cell proliferation and differentiation (2, 12). Histamine receptors have been characterized in several malignant cell types with the additional finding that they can activate multiple signaling pathways (13). Moreover, HA has been demonstrated to act as an autocrine/paracrine growth factor both \textit{in vivo} and \textit{in vitro} (14-16).

We have previously reported that HA stimulates the cAMP production via H2R inhibiting the clonogenic growth of PANC-1 cells. This antiproliferative effect is evoked by H2 agonists or by Fk, a direct activator of adenylate cyclase (3). We have also demonstrated that HA induces a partial cellular differentiation through H2R stimulation in PANC-1 cells (4). HA-induced differentiation of tumor cells through H2R activation has been reported (17, 18). Proteins related to cell proliferation and differentiation, such as c-Fos and c-Jun, have also been described as being regulated by HA via H2R in a differential manner in neoplastic cell lines (19, 20). However, our present results showed that the c-Fos level was not modulated by HA, the H2-agonist Dim or Fk suggesting that a potential linkage between c-Fos protein expression and cAMP metabolic pathway was not present.

In our current work, the antiproliferative effect induced by HA through H2R and evoked by the specific H2-agonist Dim on PANC-1 cells was clearly corroborated by a significant time-dependent decrease of the cell population growth rate. These results correlated with a lower expression of PCNA protein when cells were exposed to HA, Dim or Fk. To get insights into the mechanisms involved in this inhibitory action exerted by HA via H2R on PANC-1 cells, we studied the cell cycle and the population doubling time. Cell cycle distribution analysis revealed an increased accumulation of G0/G1 phase cells at 48 h, indicating a retardation of cell cycle progression in coincidence with a larger T-value in treated cells.

In order to study the HA-mediated G0/G1 cell arrest, we evaluated p53 protein in the PANC-1 cell line bearing mutated p53. Several lines of evidence show that the cell cycle arrest and apoptotic activities of the wild-type p53 protein are separable functions in the suppression of tumour cell growth. In addition, a series of p53 mutants retaining these activities has been recently characterized (21). Our results indicated that mutated p53 expression is not related to the G0/G1 cell accumulation induced by HA in PANC-1 cells.

The action of HA on programmed cell death is still controversial. In this regard, pro and antiapoptotic effects of HA and HA-antagonists in both normal and cancer cells have been described (22, 23). We recently reported the resistance to apoptosis induced by HA via H2R in PANC-1 cells (4). However, the molecular mechanism that renders pancreatic cancer cells resistant to HA-mediated apoptosis still remains unknown. In this work, we evaluated the alteration of the apoptotic pathway by the study of Bcl-2 proteins. It is well-known that Bcl-2 family proteins are the main regulators of the apoptotic process acting either to inhibit or to promote it. There has been much discussion on a model in which the ratio of Bcl-2 to its heterodimerization partner Bax determines survival or death, following a cell-death stimulus in normal cells and cancer cell lines carrying wild-type p53 (5, 24). Though overexpression of Bcl-2 is common in many types of human cancer, we could not detect Bcl-2 in treated and untreated PANC-1 cells. In this sense, our results are similar to other reports of absence or low expression of Bcl-2 protein in pancreatic ductal adenocarcinomas (25, 26).
It is known that Bcl-xL and Bcl-xS are isoforms encoded by two splice variants of the bcl-x gene. Bcl-xL acts as a potent inhibitor of apoptosis, while Bcl-xS is thought to antagonize Bcl-2 and Bcl-xL by binding and inactivating these apoptotic inhibitors (24). Though our findings showed an enhanced expression of Bcl-xL and Bcl-xS isoforms in PANC-1 cells receiving HA, Dim or Fk, the level of Bcl-xL expression was higher than that of Bcl-xS for each treatment. Recent reports show the ability of Bcl-xL to inhibit the entry into the cell cycle through elevation of p27 (27). A similar function of Bcl-xL in delaying HA-induced cell cycle progression via H2R might be suggested in PANC-1 cells.

Bax is a transcriptional target of the tumour suppressor p53, which is mutated in the majority of human cancers. It has been reported that Bax protein may be modulated in presence of wild-type or mutated p53 after apoptotic stimuli (28). Results from our current study demonstrated that HA, Dim and Fk diminished Bax expression and that Bax modulation was not related to the p53 level in PANC-1 cells. Taken all together, these findings indicate that cAMP levels are involved in Bcl-2 protein modulation. The relative levels of Bax, Bcl-xL and Bcl-xS might define the HA-induced resistance to apoptosis in PANC-1 cells, since the balance between the proapoptotic Bax and the antiapoptotic Bcl-xL protein was altered in HA-exposed cells. In this sense, our findings are also in agreement with other reports about the protective role of high levels of Bcl-xL protein from the induction of apoptosis by different agents in pancreatic cancer cells (28, 29).

The growth-inhibitory action of cAMP in mesenchymal and epithelial cells has been known for more than 25 years. Nevertheless, recent data suggest a role for cAMP in cell protection against apoptosis. In concordance with our results, Boucher and coworkers have reported that increased intracellular cAMP concentrations inhibit cell growth, cell cycle progression and apoptosis in the pancreatic tumour cells Mia-PaCa (30). Our findings suggest that the antiproliferative, not apoptotic, effect of HA-induced cAMP production via H2R on PANC-1 cells may involve a modulation of the Bcl-2 protein family. The alteration of this balance may allow sensitisation to the differentiating effect of HA, opening the perspectives for the potential application of HA in pancreatic cancer therapy. At present, HA is being tested in clinical trials as an adjuvant in metastatic melanoma therapy due to its well known effect on immune response (31).

In conclusion, we clearly demonstrated that HA exerts an inhibitory effect on PANC-1 cell growth via H2R activation, induces a G0/G1 cell cycle arrest slowing down the cell cycle progression and also modulates the expression of Bcl-2 family proteins.

Acknowledgements

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References


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