The role of cyclooxygenase-2 in mediating the effects of histamine on cell proliferation and vascular endothelial growth factor production in

The Role of Cyclooxygenase-2 in Mediating the Effects of Histamine on Cell Proliferation and Vascular Endothelial Growth Factor Production in Colorectal Cancer

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Abstract

Purpose: Activity of histidine decarboxylase, the key enzyme in the synthesis of histamine, has been shown to be increased in several types of human tumors. We attempted to establish whether the possible involvement of histidine decarboxylase and histamine in colorectal carcinogenesis might be mediated by the activation of the cyclooxygenase-2 (COX-2) pathway.

Experimental Design: Expression/activity of histidine decarboxylase, histamine content, and prostaglandin E_2 (PGE_2) production were analyzed in 33 colorectal cancer samples and in the HT29, Caco-2, and HCT116 colon cancer cell lines. The effects of histamine, celecoxib, and H_1, H_2, and H_4 receptor antagonists on COX-2 expression/activity, cell proliferation, and vascular endothelial growth factor (VEGF) production were assessed in the three colon cancer lines that showed different constitutive COX-2 expression.

Results: We showed the up-regulation of histidine decarboxylase protein expression and activity in the tumor specimens when compared with normal colonic mucosa. Histidine decarboxylase activity and histamine content were also significantly higher in metastatic tumors than in nonmetastatic ones. These variables significantly correlated with tumor PGE_2 production. The administration of histamine increased COX-2 expression/activity, cell proliferation, and VEGF production in the COX-2-positive HT29 and Caco-2 cells. Treatment with either H_2/H_4 receptor antagonists or celecoxib prevented these effects. Histamine had no effect on both the COX-2 pathway and VEGF production in the COX-2-negative HCT116 cells.

Conclusions: Our data showed that histamine exerts both a proproliferative and a proangiogenic effect via H_2/H_4 receptor activation. These effects are likely to be mediated by increasing COX-2-related PGE_2 production in COX-2-expressing colon cancer cells.

Histamine plays a pivotal role in a number of processes, including inflammation, allergic reaction, gastric acid secretion, and neurotransmission. These different biological effects are mediated through the activation of specific histamine membrane receptors (i.e., H_1, H_2 H_3 and H_4), which differ in their tissue expression profiles and functions (reviewed in ref. 1). The H_1 receptor is mainly expressed in the brain, endothelial cells, and smooth muscle cells and is thought to play an important role in allergy. The H_2 receptor regulates gastric acid secretion in the stomach. The H_3 receptor is mainly restricted to cells in the central nervous system and regulates, as a presynaptic autoreceptor, the release of histamine and neurotransmitters by neurons. The H_4 receptor is a new member of the histamine receptor family and is predominantly expressed in peripheral blood leukocytes. However, very little is known about the physiologic role of H_4 receptor.

Histamine levels in cells and tissues are regulated by the activity of histidine decarboxylase (EC 4.1.1.22) that is the only enzyme responsible for the generation of histamine from L-histidine (2). Therefore, histidine decarboxylase can serve as a specific marker for biosynthesis of histamine. It has been shown that levels of mRNA encoding histidine decarboxylase, histidine decarboxylase protein expression, and enzymatic activity are significantly increased in both experimental and human tumors, such as melanoma (3, 4), small cell lung carcinoma (5), breast carcinoma (6), endometrial cancer (7), and colorectal carcinoma (8). These data suggest that histamine may be directly involved in tumor development and progression. However, the most compelling data supporting a potential role of histamine in carcinogenesis are the results of clinical

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trials that showed increased survival of gastric and colon cancer patients after treatment with H_2 receptor antagonists, such as cimetidine or ranitidine (9–12).

It has been reported that histamine may regulate cell proliferation (13, 14). In particular, histamine has been shown to stimulate the *in vitro* and *in vivo* growth of both melanoma (15) and gastrointestinal cancer cells and treatment with cimetidine can reverse these effects (16). Tumor invasion and metastasis are, clinically, even more relevant than tumor growth. It is known that these processes are mainly dependent on the capability of tumor cells to adhere to endothelial cells and induce new microvessel formation. Cimetidine has been shown to inhibit HT29 colon cancer cell adhesion to human umbilical vein endothelial cells by blocking E-selectin expression (17). Histamine has also been reported to act as an angiogenic factor (18, 19) and induce vascular endothelial growth factor (VEGF) production in granulation tissue (20). However, the possible relationship between histamine and angiogenesis in cancer has not been investigated yet.

Several lines of evidence have shown that prostaglandin E_2 (PGE_2), the main product of cyclooxygenase-2 (COX-2) activity, can promote a number of molecular mechanisms involved in colorectal carcinogenesis (reviewed in ref. 21), in particular tumor cell proliferation and angiogenesis (22–25). The role of histamine in modulating PGE_2 production has been shown in experimental models of inflammation (26, 27). To our knowledge, the possible interaction between histamine and COX-2 activity in tumor cells has not been investigated yet.

In the present study, we addressed the hypothesis that activation of the COX-2 pathway mediates, at least in part, the possible link between histamine and colorectal cancer. We determined histidine decarboxylase expression/activity and histamine content in human colorectal cancer specimens. These variables were correlated with tumor staging and PGE_2 production. We also evaluated the effects of histamine and H_1, H_2, and H_4 receptor antagonists on COX-2 protein expression/enzymatic activity, cell proliferation, and VEGF production in the HT29, Caco-2, and HCT116 colon cancer cell lines that have different constitutive COX-2 protein expression.

Materials and Methods

Patients and tissue collection. Tissue samples were obtained from 33 patients (15 males and 18 females; median age, 65 years; age range, 46-81 years) who had consecutively undergone surgical resections for primary sporadic colorectal adenocarcinomas at the Department of General Surgery, University of Florence, Florence, Italy. All patients were thoroughly informed about the aims of the study and gave written consent for the investigation in accordance with the ethical guidelines of our University. Six tumors were located in the proximal colon (up to the splenic flexure), 17 in the distal colon, and 10 in the rectum. Twenty-seven tumors were classified as adenocarcinomas and six tumors were classified as mucinous carcinomas (when >50% of the tumor volume was composed of mucin). Adenocarcinomas were classified as well differentiated ($n = 0$), moderately differentiated ($n = 24$), and poorly differentiated ($n = 3$). Tumors were classified into four stages according to the American Joint Committee on Cancer staging system (28): stage I (T_1-T_2, N_0, and M_0; $n = 3$), stage II (T_3-T_4, N_0, and M_0; $n = 16$), stage III (any T, N_{1-2}, M_0; $n = 12$), and stage IV (any T and any N and M_1; $n = 2$).

Cancer tissue (from the edge of the tumor) and adjacent normal mucosa (at least 10 cm from the tumor) were excised from each surgical specimen. The samples were washed in PBS. They were flash-frozen in liquid nitrogen for reverse-transcriptase PCR, frozen at −80°C for Western blot analysis, and frozen at −20°C for histidine decarboxylase activity and histamine and PGE_2 production evaluation until processing. Other samples were fixed in 4% formaldehyde and embedded in paraffin for immunohistochemical analysis.

Cell culture and drugs. Experiments were done on the HT29, Caco-2, and HCT116 human colon cancer cell lines and on the HL-60 human leukemia cell line. The HT29 and HCT116 cells were a gift from Dr. Claudia Casini Raggi (Department of Clinical Physiopathology, University of Florence). The Caco-2 and HL-60 cells were purchased from Interlab Cell Line Collection (Genoa, Italy). Both HT29 and Caco-2 cells constitutively express COX-2 protein (29, 30), whereas the HCT116 cells do not express COX-2 (29). The HL-60 cells constitutively express a high level of the H_1, H_2, and H_4 receptors (31, 32). These cells served as a positive control. The cells were cultured as previously described (25). Histamine dihydrochloride, the histamine H_1 receptor antagonist mepyramine maleate, and the histamine H_2 receptor antagonist zolantidine were purchased from R.B.I. (Natick, MA). The H_4 receptor antagonist JNJ 7777120 was supplied by Johnson & Johnson Pharmaceutical Research and Development LLC (San Diego, CA). The selective COX-2 inhibitor celecoxib was provided by Monsanto (St. Louis, MO).

Histidine decarboxylase immunostaining. Four-micrometer-thick sections were cut from the formalin-fixed and paraffin-embedded tissue blocks and processed as previously described (24). Immunohistochemical staining was done using the streptavidin-biotin peroxydase method. A rabbit polyclonal antibody (Euro-Diagnostica AB, Malmö, Sweden) for histidine decarboxylase at 1:1,000 dilution at 4°C overnight was used. Positive control for histidine decarboxylase immunostaining included sections of normal human gastric mucosa of the fundus. Tissue sections, treated with nonimmune rabbit serum in place of the primary antibodies were used as negative controls. Observations were carried out with a Reichert-Jung Microstar IV light microscope (Cambridge Instruments, Buffalo, NY) and registered with a video camera (WPI, Sarasota, FL) interfaced with a personal computer through a Matrox Marvel G400-TV digitizing card (Matrox Graphics, Dorval, Canada).

Two pathologists (L.M. and D.B.) independently evaluated the immunostained specimens. The extent of histidine decarboxylase immunostaining was recorded semiquantitatively using a three-grade system, based on the percentage of stained tumor epithelial cells: grade 0, 1% to 20%; grade 1, 21% to 70%; grade 2, >70%.

Assay for histidine decarboxylase activity. Histidine decarboxylase activity was assayed by measuring $[^{14}C]CO_2$ evolved from L-[carboxyl-^{14}C]-histidine (46 mCi mmol^{-1}) according to the method described previously (33). Determinations were done in quintuplicate. The enzyme activity is expressed as ng of histamine formed per either μg or mg of protein per hour in the tissue samples and in the cells, respectively.

Histamine content determination. Histamine content was assessed fluorimetrically in tissue specimens and in the cells using the method of Shöre et al. (34) as modified by Kremzner and Wilson (35). Histamine was extracted from tissue and cell homogenates as previously reported (36) and derivatized to a fluorogenic compound with *ortho*-phthaldialdehyde. Fluorescence was monitored at the emission/excitation ratio of 365:455 nm using a spectrofluorimeter Shimadzu RF 5000 (Kyoto, Japan). The detection limit of the method is 10 pg histamine base mL^{-1}. The authenticity of histamine in tissue specimens and in the cells was shown by recording the excitation and emission fluorescent spectra. Incubation of extracted tissue samples with histaminase (1 mg mL^{-1} from porcine kidney) selectively removed the histamine peak, confirming its identity. Determinations were done in quintuplicate. Histamine values were expressed as ng per either μg or mg of protein in the tissue samples and the cells, respectively.

Prostaglandin E_2 measurement. Normal mucosa and tumor samples were processed as described previously (24). Supernatants of the HT29,

Caco-2, and HCT116 cells were prepared according to the method described previously (25). Drugs (1 μmol/L mepyramine, zolantidine, JNJ 7777120, and celecoxib) were added 30 minutes before histamine (1 μmol/L) administration. After 24 hours of incubation, the supernatants were collected. Supernatants (500 mL) of tissue homogenates and 100 μL supernatants of the cells were used for PGE_2 determination using a competitive enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) according to the method described by Pradelles et al. (37). Protein concentration in the tissue samples and in the cells was determined according to the method described by Lowry et al. (38). Bovine serum albumin was used as the standard. Determinations were done in quintuplicate. PGE_2 values were expressed as μg per mg protein in the tissue samples and pg per μg protein in the cells.

Reverse-transcriptase PCR. RT-PCR was done on total RNA extracted from tissue samples and cells using the RNAeasy Kit (Qiagen, Hilden, Germany). Two micrograms of total RNA were retrotranscribed to cDNA with the Improm-II Reverse Transcriptase kit (Promega, Madison, WI) and amplified with the following primers for the human H_1, H_2, and H_4 histamine receptor subtypes: 5′-AAGTCACCATCCCAAACCCCCAAG-3′/5′-TCAGGCCCTGCTCATCTGTCTTGA-3′ for the H_1 receptor, 5′-AGGAACGAGACCAGCAAGGGCAAT-3′/5′-GGTGGCTGCCTTCCAGGAGCTAAT-3′ for the H_2 receptor, and 5′-CCGTTTGGGTGCTGGCCTTCTTAG-3′/5′-TCCACCACCCTGTTGCTGCTGTA-3′ for the H_4 receptor. The sizes of the three RT-PCR products were 195, 197, and 203 bp, respectively. A glyceraldehyde-3-phosphate dehydrogenase 196-bp PCR product was obtained using the following primer pair: 5′-CCATGGAGAAGGCTGGGG-3′/5′-CAAAGTTGTCATGGATGACC-3′. Amplification of cDNA was done with the following profiles: 95°C for 15 minutes followed by 40 cycles at 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds for histamine receptors and 95°C for 2 minutes followed by 30 cycles at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 40 seconds for glyceraldehyde-3-phosphate dehydrogenase. The HL-60 cells served as a positive control for histamine receptors.

Western blot analysis. Total proteins from tumor tissue and the corresponding normal mucosa were obtained as described previously (24). The HT29, Caco-2, and HCT116 cells were grown to subconfluence and starved for 24 hours in 0.1% FCS-supplemented medium. After incubation with 1 μmol/L histamine in the absence or presence of drugs for indicated times, cells were washed in PBS and lysed with radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 7.1) 150 mmol/L NaCl, 5 mmol/L EDTA, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol]. Seventy micrograms of total proteins, as evaluated by bicinchoninic acid assay, from tissue or cultured cells were subjected to Western blotting and immunoblotting analysis as previously described (25). The loading and transfer of equal amounts of proteins were ascertained by either reblotting the membrane with an anti-actin antibody for tissue samples or staining the membrane with Ponceau S for cell samples. Primary antibodies used were anti-histidine decarboxylase rabbit polyclonal antibody (1:1,000; Euro-Diagnostica), anti-COX-2 goat polyclonal antibody (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-VEGF mouse monoclonal antibody (1:1,000, Santa Cruz Biotechnology), and goat polyclonal anti-actin antibody (1:1,000, Santa Cruz Biotechnology). Binding of each primary antibody was determined by addition of suitable peroxidase-conjugated secondary antibodies (1:5,000; Amersham, Braunschweig, Germany).

Cell viability assay. The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay was done to determine the cytotoxicity of mepyramine, zolantidine, JNJ 7777120, and celecoxib against the HT29, Caco-2, and HCT116 cells according to the method previously described (25).

Cell proliferation assay. HT29, Caco-2, and HCT116 cell proliferation was determined by the [^{3}H]thymidine incorporation assay according to the method previously described (25). The experiments were done in quintuplicate, and the values were expressed as dpm per well.

Quantification of vascular endothelial growth factor production. An ELISA method was used to quantify the secreted VEGF in the culture medium of the HT29, Caco-2, and HCT116 cells according to the method previously described (25). Determinations were done in quintuplicate. VEGF values were expressed as pg per μg protein.

Statistical analysis. Histidine decarboxylase activity, incorporation of [^{3}H]thymidine, and histamine, PGE_2, and VEGF levels were expressed as mean values ± SE. The relationships among histidine decarboxylase activity, histamine content, and PGE_2 production were evaluated using the Spearman correlation coefficients (r_s). Differences in histidine decarboxylase activity and histamine content in tumor and normal mucosa specimens and in tumors with and without metastases were analyzed using the Mann-Whitney test. [^{3}H]thymidine incorporation, PGE_2, levels and VEGF levels in the cells were compared using the paired-value Wilcoxon test or the Mann-Whitney test, as appropriate. Statistical analysis was done using Stata Statistic Software (release 5.0; Stata Corp., College Station, TX). All of the *P*s resulted from the use of two-sided statistical tests; $P < 0.05$ were considered statistically significant.

Results

Histidine decarboxylase expression and activity in human colorectal cancer and colon cancer cells. Most of the tumors showed extensive immunostaining for histidine decarboxylase protein: 10 tumors (30.3%) were grade 0, 11 (33.3%) were grade 1, and 12 (36.4%) were grade 2. Histidine decarboxylase was found mainly in the tumor epithelial cells (Fig. 1A). There was also some staining of the inflammatory mononuclear cells (i.e., histamine-producing mast cells and macrophages) that infiltrate the tumors. However, the extent of histidine decarboxylase expression by nonepithelial interstitial cells never exceeded that found in the corresponding tumor tissue. Histidine decarboxylase staining was not detectable in normal colon epithelial cells (Fig. 1B). Histidine decarboxylase expression was occasionally found in the histaminergic enteroendocrine cells (i.e., enterochromaffin-like cells) and in nonepithelial interstitial cells of normal mucosa.

Western blot analysis confirmed the overexpression of histidine decarboxylase protein that had been detected by immunohistochemistry. A higher amount of protein was found in the neoplastic tissue when compared with the corresponding normal mucosa (Fig. 1C). The main form of histidine decarboxylase protein detected in our tumor specimens had a molecular mass of 74 kDa (Fig. 1C). Low levels of histidine decarboxylase protein were detected in normal mucosa of 25% to 30% of the specimens. This may reflect the abovementioned enterochromaffin-like cell and nonepithelial cell histidine decarboxylase expression. However, the level of histidine decarboxylase in normal mucosa never exceeded that found in the neoplastic tissue. Histidine decarboxylase protein expression was also found in the HT29, Caco-2, and HCT116 colon cancer cells. Histidine decarboxylases (74- and 54 kDa) gave bands of similar intensity (Fig. 1D).

Histidine decarboxylase activity and histamine content were significantly higher in the tumor specimens than in the corresponding normal mucosa (42.7 ± 2.4 versus 15.8 ± 1.0 ng of histamine/μg protein/h, $P = 0.003$ and 28.1 ± 2.4 versus 0.7 ± 0.3 ng/μg protein, $P < 0.0001$, respectively; Fig. 1E and F). These variables were also significantly higher in tumors with lymph node and/or distant metastases (stages III-IV) than in those without any metastases (stages I-II) (51.51 ± 3.1 versus 35.1 ± 2.7 ng of histamine/μg protein/h, $P < 0.001$ and

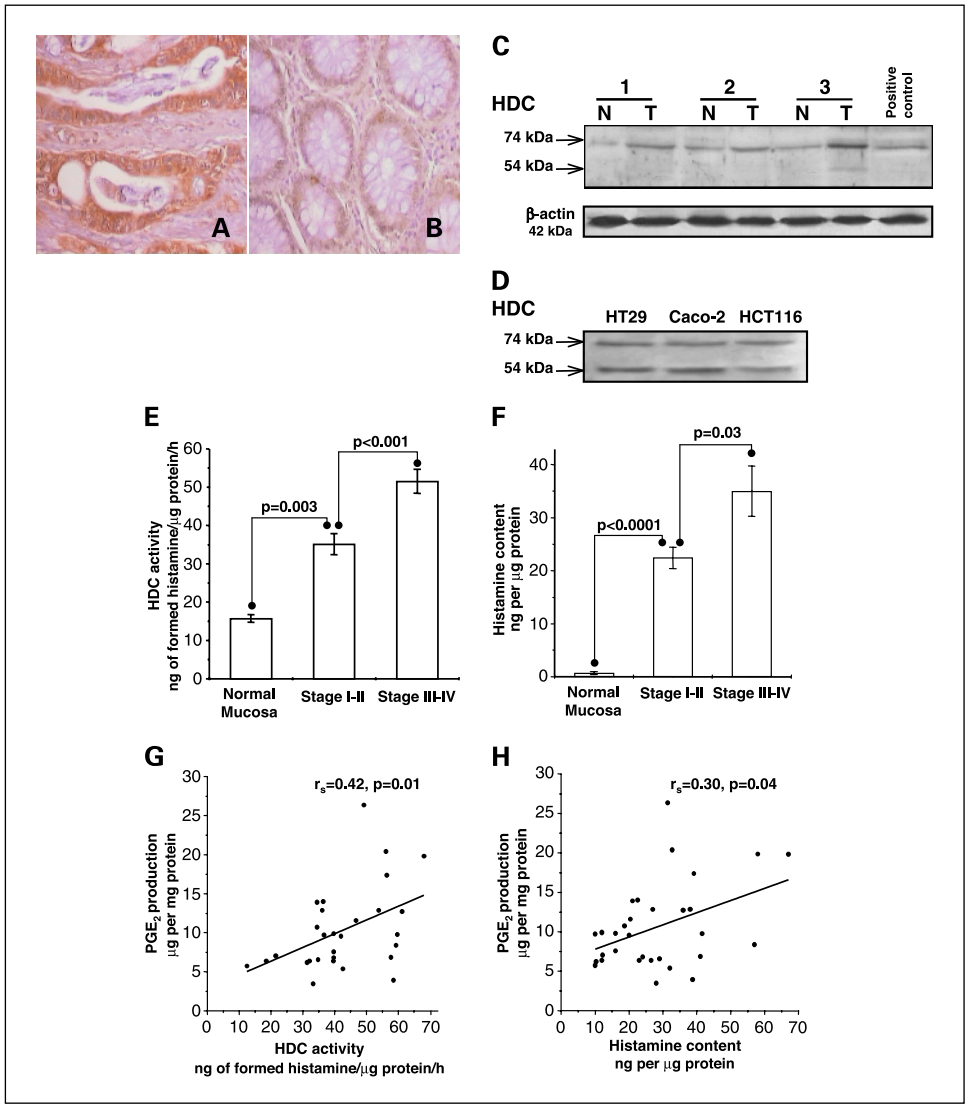

Fig. 1. Histidine decarboxylase (*HDC*) protein expression and activity in human colorectal cancer specimens. *A*, immunohistochemical analysis showed that tumor epithelial cells were positive for histidine decarboxylase staining. Hematoxylin counterstain (original magnification, ×25). *B*, histidine decarboxylase expression was not detectable in the normal colon epithelial cells. Hematoxylin counterstain (original magnification, ×25). *C*, Western blot analysis. Expression of histidine decarboxylase in representative paired adenocarcinoma and adjacent normal mucosa from three patients. A higher amount of histidine decarboxylase was found in the neoplastic tissue than in the normal mucosa. Histidine decarboxylase (74 kDa) was the main form detected in the colorectal cancer specimens. Human gastric mucosa of the fundus served as a positive control for histidine decarboxylase. Samples were normalized for protein loading (70 μg) by reblotting the membrane-bound protein with an anti-actin antibody. Abbreviations: T, tumor; N, normal mucosa. *D*, Western blot analysis. Expression of histidine decarboxylase in the HT29, Caco-2, and HCT116 colon cancer cells. Histidine decarboxylases (74- and 54 kDa) gave bands of equal intensity. Samples were normalized for protein loading (70 μg) by Ponceau S staining of the membrane-bound protein. *E* and *F*, histidine decarboxylase activity and histamine content were significantly higher in the 33 tumor samples than in the corresponding normal mucosa specimens. They were also significantly higher in the 19 metastatic (stages III and IV) tumors than in the 14 nonmetastatic (stages I and II) ones. Columns, means of five determinations; bars, SE. *G* and *H*, histidine decarboxylase activity and histamine content were significantly correlated with PGE_2 production.

35.0 ± 3.1 versus 22.4 ± 2.0 ng/μg protein, $P = 0.03$, respectively; Fig. 1E and F). PGE_2 production was significantly higher in the tumor specimens than in the corresponding normal mucosa (10.3 ± 2.0 versus 3.1 ± 0.5 μg/mg protein, $P < 0.0001$). It was also significantly higher in tumors with lymph node and/or distant metastases (stages III-IV) than in those without any metastases (stages I-II) (11.5 ± 3.0 versus 7.8 ± 1.8 μg/mg protein, $P = 0.02$). Histidine decarboxylase activity and histamine content were significantly correlated with PGE_2 production ($r_s = 0.42$, $P = 0.01$ and $r_s = 0.30$, $P = 0.04$, respectively; Fig. 1G and H).

Histidine decarboxylase activity values in the HT29, Caco-2, and HCT116 cells were 11.5 ± 5.7, 9.7 ± 6.3 and 15.3 ± 4.5 ng of histamine/mg protein/h, respectively. Total histamine levels (in the cells plus supernatant) were 2.7 ± 0.9, 3.5 ± 0.2, and 8.4 ± 1.2 ng/mg protein in the HT29, Caco-2, and HCT116 cells, respectively. Our *in vitro* results confirmed that histamine could be synthesized *de novo* by colon cancer cells. To confirm the authenticity of histidine decarboxylase activity, we verified the suppression of the histamine forming capacity in the cells by preincubating some cell extracts with 10^{-5} mol/L α-fluoromethylhistidine, a selective inhibitor of histidine decarboxylase (data not shown).

Expression of H_1, H_2, and H_4 receptor mRNA in human colorectal cancer and colon cancer cells. RT-PCR detected mRNA levels for H_1, H_2, and H_4 receptors in both colorectal cancer specimens and adjacent normal colonic mucosa. (Fig. 2A). mRNA of the three types of receptor was also detected in the COX-2-positive HT29 and Caco-2 cells and in the COX-2-negative HCT116 cells (Fig. 2B and C, respectively).

Effects of histamine receptor antagonists and celecoxib on cell viability. Drug cytotoxicity was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Mepyramine, zolantidine, JNJ 7777120, and celecoxib had similar cytotoxic effects against the HT29 and Caco-2 cells with significant loss of viability at concentrations of ≥10 μmol/L (data not shown). A significant loss in cell viability in the HCT116 cells was observed after treatment with mepyramine, zolantidine, JNJ 7777120, or celecoxib concentrations of ≥25 μmol/L (data not shown). Subsequent experiments were done using noncytotoxic doses of both histamine receptor antagonists and celecoxib (1 μmol/L).

Effects of histamine on cyclooxygenase-2 expression and activity in the HT29, Caco-2, and HCT116 colon cancer cells. Western blot analysis showed a significant basal COX-2 expression in the HT29 cells (Fig. 3A and D), whereas very low basal levels were present in the Caco-2 cells (Fig. 3B and E). No detectable basal COX-2 expression was present in the HCT116 cells (Fig. 3C). Treatment of the HT29 and Caco-2 cells with histamine increased COX-2 protein expression in a time-dependent manner (Fig. 3A and B). COX-2 protein levels reached a maximum at 18 hours in both cell lines and then declined (Fig. 3A and B). Treatment of the HCT116 cells with histamine did not cause any increase in COX-2 expression (Fig. 3C). To determine which subtype/s of histamine receptor is/are involved in COX-2 activation, the HT29 and Caco-2 cells were treated with histamine in the presence of selective antagonists to H_1 (mepyramine), H_2 (zolantidine), or H_4 (JNJ 7777120). Zolantidine or JNJ 7777120 but not mepyramine prevented COX-2 induction by histamine (Fig. 3D and E).

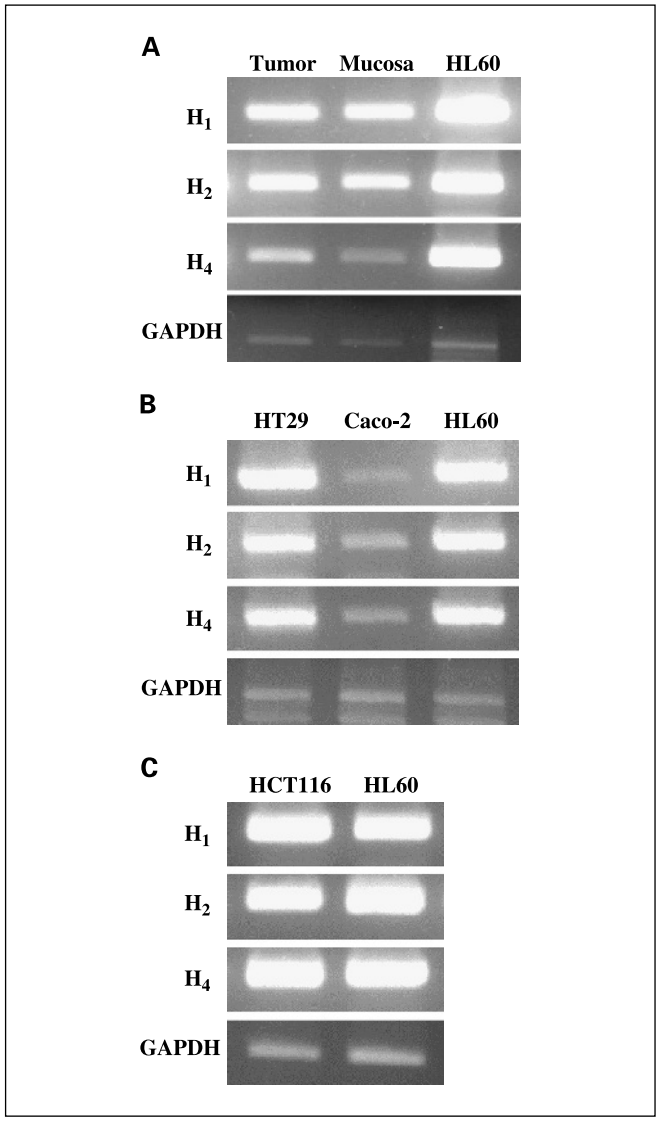

Fig. 2. RT-PCR. *A*, mRNA levels for H_1, H_2, and H_4 receptors were detected in tumor tissue and in corresponding normal colon mucosa. *B* and *C*, mRNA for the three histamine receptor subtypes was also found in both the COX-2-positive HT29 and Caco-2 human colon cancer cell lines and the COX-2-negative HCT116 human colon cancer cell line. The HL60 human leukemia cell line was used a positive control for H_1, H_2, and H_4 receptors. The housekeeping gene *GAPDH* was used as an internal control.

Basal PGE_2 production did not significantly differ among the three cell lines. After incubation of the cells with histamine, we found a significant increase in PGE_2 concentrations in the supernatants of the HT29 and Caco-2 cells but not in those of the HCT116 cells (Table 1). The administration of celecoxib significantly reduced histamine-mediated PGE_2 production in the HT29 and Caco-2 cells (Table 1). This finding suggests that COX-2 was the main source of PGE_2 after histamine treatment. The administration of zolantidine or JNJ 7777120 significantly reduced the histamine-stimulated production of PGE_2 (Table 1). Mepyramine failed to affect PGE_2 levels after histamine administration (Table 1). Combination treatment with zolantidine and JNJ 7777120 determined an additive effect in reducing histamine-mediated increase in PGE_2 production (Table 1).

Effects of histamine on HT29, Caco-2, and HCT116 cell proliferation. We examined the effect of histamine on the proliferation of the HT29, Caco-2, and HCT116 cells by measuring the incorporation of [^{3}H]thymidine to assess DNA synthesis. Basal proliferation did not differ among the three cell lines and it was not affected by treatment with mepyramine, zolantidine, JNJ 7777120, or celecoxib (Fig. 4). The administration of histamine stimulated cell proliferation in all cell lines (Fig. 4). The addition of mepyramine did not affect the proliferative response of the cells to histamine (Fig. 4). On the contrary, the histamine-induced stimulation of HT29, Caco-2, and HCT116 cell proliferation was significantly antagonized by the H_2 and H_4 receptor antagonists zolantidine and JNJ 7777120, respectively. Treatment with the COX-2 inhibitor celecoxib suppressed the growth-promoting effect of histamine in the HT29 and Caco-2 cells, but it had no effect in the HCT116 cells (Fig. 4). Combination treatment with zolantidine and JNJ 7777120 determined an additive effect in reducing histamine-stimulated proliferation in the three cell lines (Fig. 4).

Effects of histamine on vascular endothelial growth factor levels in the HT29, Caco-2, and HCT116 colon cancer cells. To examine the effect of histamine on tumor angiogenesis, we evaluated VEGF production in the HT29, Caco-2, and HCT116 cells. Western blot analysis showed basal VEGF expression in the HT29 and HCT116 cells (Fig. 5A and C), whereas no detectable basal signal was present in the Caco-2 cells (Fig. 5B). Treatment of the HT29 and Caco-2 cells with histamine increased VEGF protein levels in a time-dependent manner with a maximum at 18 and 24 hours in the two cell lines, respectively (Fig. 5A and B). Treatment of the HCT116 cells with histamine did not cause any increase in VEGF expression (Fig. 5C). Histamine-mediated VEGF induction in the HT29 and Caco-2 cells was prevented by the administration of zolantidine, JNJ 7777120, or celecoxib (Fig. 5D and E), whereas mepyramine had no effect (Fig. 5D and E).

Quantitative determination of VEGF levels confirmed Western blot analysis results. Basal VEGF production was significantly higher in the HCT116 cells than in HT29 (734.1 $\pm$ 31.5 versus 604.5 $\pm$ 23.4 pg/μg protein, $P = 0.03$) and Caco-2 ones (734.1 $\pm$ 31.5 versus 450.7 $\pm$ 12.5 pg/μg protein, $P = 0.02$). Treatment of the cells with histamine led to a significant increase in VEGF levels in the HT29 and Caco-2 cells, whereas

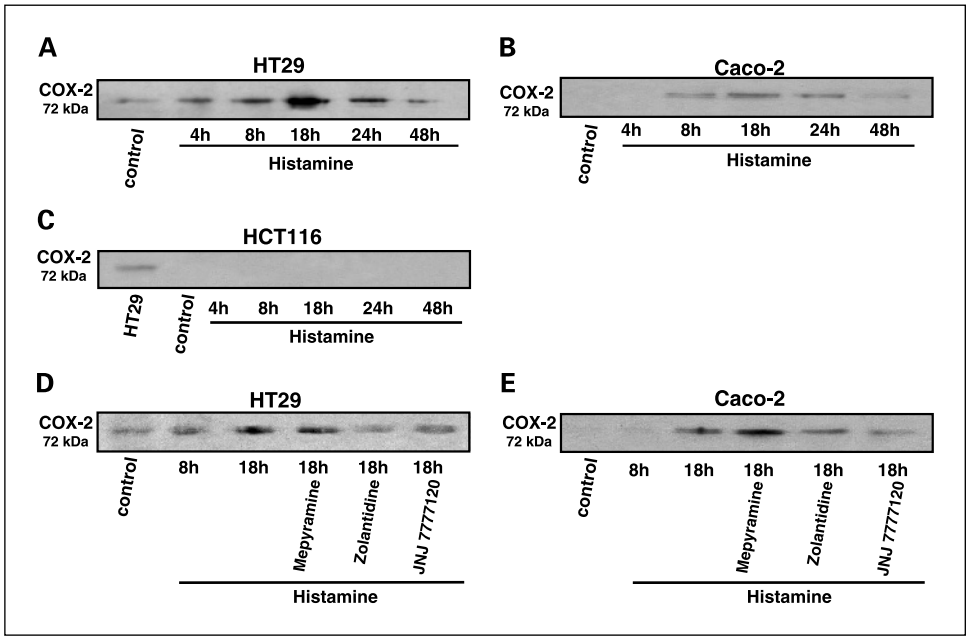

Fig. 3. Western blot analysis. Effects of histamine on COX-2 protein expression in the HT29, Caco-2, and HCT116 colon cancer cells. *A* and *B*, treatment with histamine (1 μmol/L) increased COX-2 protein levels in a time-dependent manner in the HT29 and Caco-2 cells. Up-regulation of COX-2 was maximum after 18 hours. *C*, the same treatment had no effect in the HCT116 cells. *D* and *E*, the H_2 receptor antagonist zolantidine (1 μmol/L) and the H_4 receptor antagonist JNJ 7777120 (1 μmol/L) prevented the histamine-induced overexpression of COX-2 in the HT29 and Caco-2 cells. The H_1 receptor antagonist mepyramine had no effect on histamine-induced COX-2 overexpression. Samples were normalized for protein loading (70 μg) by Ponceau S staining of the membrane-bound protein.

the same treatment had no effect in the HCT116 cells (Table 1). The administration of zolantidine, JNJ 7777120, or celecoxib to the HT29 and Caco-2 cells significantly reduced the histamine-stimulated production of VEGF (Table 1). The administration of mepyramine did not affect VEGF levels (Table 1). Combination treatment with zolantidine and JNJ 7777120 had an additive effect on reducing the histamine-mediated increase in VEGF production (Table 1).

Discussion

Although the hypothesis that histamine might be involved in carcinogenesis was proposed in the 1960s (39), it still remains under discussion today. Accumulated evidence points to a direct relationship between up-regulation of histidine decarboxylase activity and growth of several types of human tumors (3–7). As regards colorectal cancer, Garcia-Caballero et al. (8) have shown a significantly higher histidine decarboxylase activity in 10 tumor specimens than in the corresponding normal colon mucosa. More recently, Boér et al. (40) have shown a strong immunoreactivity for histidine decarboxylase in both adenomatous polyps and colorectal cancer specimens. Our results showed a sustained histidine decarboxylase protein expression and activity in both human colorectal tumor specimens and three colon cancer cell lines (i.e., HT29, Caco-2, and HCT116). We also showed the expression of H_1, H_2, and

Table 1. Effects of histamine, celecoxib, and H_1, H_2, and H_4 receptor antagonists on PGE_2 (pg/μg protein) and VEGF (pg/μg protein) production in the HT29, Caco-2, and HCT116 colon cancer cell lines

Drugs	HT29 cell line		Caco-2 cell line		HCT116 cell line	
	PGE_2 production	VEGF production	PGE_2 production	VEGF production	PGE_2 production	VEGF production
None	19.8 ± 1.8	604.5 ± 23.4	12.5 ± 1.7	450.7 ± 12.5	16.9 ± 2.0	734.1 ± 31.5
Mepyramine (1 μmol/L)	20.3 ± 0.9	795.0 ± 19.9	10.4 ± 0.4	447.0 ± 13.7	18.9 ± 0.2	731.2 ± 54.4
Zolantidine (1 μmol/L)	18.3 ± 0.7	640.4 ± 35.4	12.1 ± 2.7	462.7 ± 16.3	16.0 ± 3.1	708.3 ± 31.5
JNJ 7777120 (1 μmol/L)	18.4 ± 2.1	703.3 ± 31.3	11.8 ± 2.8	440.1 ± 22.7	20.0 ± 0.4	822.5 ± 36.8
Celecoxib (1 μmol/L)	18.9 ± 0.8	712.0 ± 36.7	12.8 ± 2.3	454.4 ± 24.2	18.4 ± 1.1	748.2 ± 71.4
Histamine (1 μmol/L)	118.7 ± 2.86*	1446.7 ± 59.8*	136.7 ± 2.6*	1043.2 ± 65.5*	19.1 ± 0.9	831.1 ± 14.2
Histamine + celecoxib	71.8 ± 1.2†	894.3 ± 14.3†	81.7 ± 2.8†	667.0 ± 36.4†	—	—
Histamine + mepyramine	109.4 ± 2.0	1165.9 ± 27.2	137.9 ± 1.7	1004.8 ± 93.6	—	—
Histamine + zolantidine	52.6 ± 1.1†	789.9 ± 31.2†	104.6 ± 3.9†	834.4 ± 19.3†	—	—
Histamine + JNJ 7777120	63.1 ± 2.5†	862.1 ± 5.7†	115.4 ± 4.3†	838.9 ± 9.2†	—	—
Histamine + zolantidine + JNJ 7777120	40.6 ± 1.0†	709.9 ± 21.3†	93.4 ± 4.1†	699.4 ± 27.6†	—	—

NOTE: Results are expressed as means ± SE of five determinations per treatment.
*$P < 0.05$, significant increase compared with unstimulated cells (Wilcoxon test).
†$P < 0.05$, significant inhibition compared with histamine treatment (Wilcoxon test).

H_4 receptor mRNA in the same tumor samples and cells. The coexistence of endogenously produced histamine and its specific membrane receptors strongly suggests an autocrine loop for histamine in colorectal cancer carcinogenesis. Immunoblot analysis showed that the main form of histidine decarboxylase protein detected in our cancer specimens had a molecular mass of 74 kDa. These findings are in contrast with those reported by previously published studies on the correlation between the immunoreactive forms of histidine decarboxylase and its enzymatic activity in mice and rats. It has been shown that the 74-kDa histidine decarboxylase is an enzymatically inactive proenzyme, which has to be activated by posttranslational processing into the 54-kDa subunit form (41, 42). Nevertheless, Yatsunami et al. (43) have shown that 74- and 54-kDa histidine decarboxylases exhibit equivalent histamine-synthesizing activity in humans. These authors hypothesized that the activity of 74-kDa histidine decarboxylases might differ in humans, mice, and rats, because their COOH-terminal regions are not completely homologous. This may explain the association between high levels of histidine decarboxylase activity and up-regulation of the 74-kDa histidine decarboxylase in our colorectal cancer specimens. Unlike with tumor tissue, we found that both the 54- and 74-kDa histidine decarboxylases are equally represented in all three colon cancer cell lines. Because multiple forms of histidine decarboxylase have been shown to be generated by tissue- or cell-specific posttranslational processing (42, 44), it might be hypothesized that different processing pathways are responsible for the different expression of the 54-kDa subunit form in either the tumor tissue or the colon cancer cell lines.

Our study showed that high levels of histidine decarboxylase activity and histamine content were correlated with the presence of lymph node and/or distant metastases in colorectal cancer. Interestingly, we also found a significant correlation between these variables and tumor PGE_2 production. A large body of experimental evidence has shown the close involvement of COX-2 activity and thus PGE_2 production in tumor proliferation and progression (reviewed in ref. 21). Collectively, our data strongly suggest a role for endogenously synthesized histamine in the acquisition of an invasive and metastatic phenotype of tumor cells. However, the specific mechanisms by which histidine decarboxylase and histamine are involved in cancer progression are still unknown.

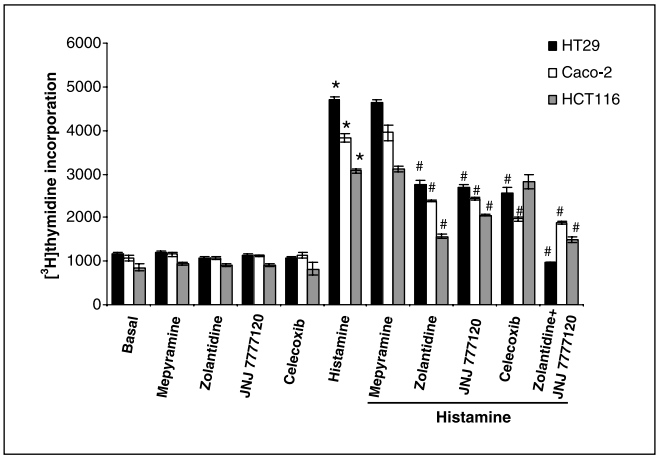

Fig. 4. Effects of histamine on HT29, Caco-2, and HCT116 cell proliferation evaluated by [^{3}H]thymidine incorporation. Basal proliferation did not differ among the three cell lines and it was not affected by treatment with mepyramine (1 μmol/L), zolantidine (1 μmol/L), JNJ 7777120 (1 μmol/L), or celecoxib (1 μmol/L). The administration of histamine (1 μmol/L) determined a stimulation of cell proliferation in the three cell lines. Mepyramine had no effect on histamine-induced increase in cell proliferation, whereas zolantidine and JNJ 7777120 prevented the proproliferative effect of histamine in the three cell lines. Celecoxib inhibited the growth-promoting effect of histamine in the HT29 and Caco-2 cells, but it had no effect in the HCT116 cells. Combination treatment with zolantidine and JNJ 7777120 determined an additive effect in reducing histamine-stimulated proliferation in the three cell lines. *Columns,* means of five different experiments; bars, SE. [^{3}H]thymidine incorporation was expressed as dpm per well. *, $P < 0.05$, significant increase compared with unstimulated cells. #, $P < 0.05$, significant inhibition compared with histamine treatment.

We showed for the first time that exogenous histamine could increase the expression of COX-2 protein in the HT29 and Caco-2 human colon cancer cells. Results from RT-PCR indicated that H_1, H_2, and H_4 receptor genes were expressed in both cell lines. However, only the selective H_2 receptor antagonist zolantidine and the selective H_4 receptor antagonist JNJ 7777120 prevented histamine-induced COX-2 overexpression, whereas the selective H_1 receptor antagonist mepyramine did not have this suppressive effect. Therefore, activation of the H_2 and H_4 receptors is likely to mediate COX-2 overexpression by histamine in the HT29 and Caco-2 cells. Interestingly, there was no observable effect of histamine on COX-2 protein levels in the HCT116 colon cancer cells, although these cells expressed H_1, H_2, and H_4 receptors. Because it has been reported that the HCT116 cells do not constitutively express COX-2, it might be hypothesized that histamine-mediated COX-2 regulation may depend on signaling pathways that are already active just at the basal levels in colon cancer cells.

We also showed that COX-2 induction by histamine resulted in a sustained enhancement of COX-2 enzymatic activity. The administration of exogenous histamine to the HT29 and Caco-2 cells determined an increase in PGE_2 production. The selective inhibition of COX-2 by celecoxib significantly reduced the histamine-induced production of PGE_2. This finding suggests that COX-2 was the main source of PGE_2 after treatment with histamine. The increase in COX-2 activity was prevented by treatment with zolantidine and JNJ 7777120, given alone or in combination, whereas mepyramine had no suppressive effect. This finding confirmed that only H_2 and H_4 receptors are functionally involved in the histamine-stimulated COX-2 activation and PGE_2 production. According to Western blot analysis results, histamine could not increase PGE_2 production in the HCT116 cells.

Previous studies showed that concentrations of exogenous histamine between 10^{-6} and 10^{-8} mol/L produce a stimulatory effect on the growth of both melanoma (4, 15) and gastrointestinal cancer cells (16, 45). Moreover, cimetidine and other H_2 receptor antagonists have been shown to prevent histamine-mediated increase in tumor cell proliferation but not affect the basal growth of the same cells in the absence of histamine (16, 45, 46). Our results confirmed the effect of 1 μmol/L exogenous histamine in stimulating proliferation of the HT29, Caco-2, and HCT116 cells. We found that both zolantidine and JNJ 7777120 prevented the cell growth–promoting activity of histamine in the three cell lines, whereas mepyramine had no effect on the histamine-induced increase in tumor cell proliferation. We also showed that zolantidine and JNJ 7777120 did not significantly affect the basal growth of the cells. It is likely that the histamine levels produced by the cells under our assay conditions are not effective in sustaining basal cell proliferation.

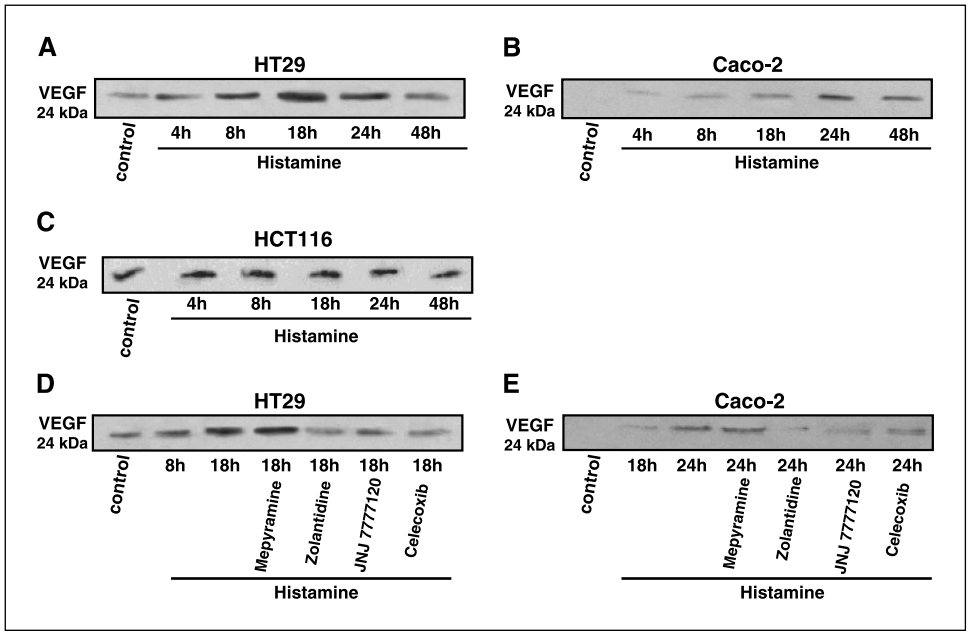

Fig. 5. Western blot analysis. Effects of histamine on VEGF protein expression in the HT29, Caco-2, and HCT116 colon cancer cells. *A* and *B*, treatment with histamine (1 μmol/L) increased VEGF protein levels in a time-dependent manner in the HT29 and Caco-2 cells. Induction of VEGF was maximum after 18 hours in the HT29 cells and after 24 hours in the Caco-2 ones. *C*, the same treatment had no effect in the HCT116 cells. *D* and *E*, the H_2 receptor antagonist zolantidine (1 μmol/L), the H_4 receptor antagonist JNJ 7777120 (1 μmol/L), and the COX-2 inhibitor celecoxib (1 μmol/L) prevented the histamine-induced overexpression of VEGF in the HT29 and Caco-2 cells. The H_1 receptor antagonist mepyramine had no effect on histamine-induced VEGF overexpression. Samples were normalized for protein loading (70 μg) by Ponceau S staining of the membrane-bound protein.

Interestingly, we showed that the proproliferative effect of histamine was inhibited by celecoxib in the HT29 and Caco-2 but not in the HCT116 cells. Collectively, our findings strongly suggest that histamine is involved in colon cancer cell proliferation via activation of both H_2 and H_4 receptors. Moreover, it is most likely that histamine exerts its proproliferative effect mainly through the up-regulation of COX-2 activity in those cancer cells that constitutively express this enzyme, whereas other mechanisms independent of the COX-2 pathway may be involved in the COX-2 negative cells. One possible mechanism by which histamine can directly lead to cell proliferation has been proposed by Wang et al. (14). These authors have shown that activation of human H_2 receptor stimulates the growth of HEK-293 cells through the histamine-induced transcriptional activation of the proto-oncogene c-*fos*.

Another mechanism potentially involved in the tumor-promoting effect of histamine is stimulation of tumor angiogenesis. Histamine has been reported to be a potent proangiogenic factor in some inflammatory models (39, 47). Ghosh et al. (20) have shown that histamine contributes to VEGF protein overexpression and angiogenesis in the carrageenin-induced granulation tissue in rats via the H_2 receptor–cyclic AMP-protein kinase A pathway. Moreover, the H_2 receptor antagonists cimetidine and roxatidine have been found to exert their suppressive effect on growth of Colon 38 tumor cell implants in mice by inhibiting VEGF protein expression and thus reducing tumor vascularization (48). Our findings showed that treatment of the COX-2-positive HT29 and Caco-2 cells with histamine determined a significant increase in VEGF levels and this effect was suppressed by the administration of zolantidine, JNJ 7777120, or celecoxib. On the contrary, no increase in VEGF levels was found in the COX-2-negative HCT116 cells after treatment with histamine. Collectively, these findings suggest that histamine-mediated increase in VEGF production mainly depends on whether histamine can also contemporarily up-regulate the COX-2 pathway via H_2/H_4 receptor activation in the same cancer cells. Because PGE_2 is known to be a potent stimulator of VEGF release (23, 25), we concluded that enhancement of COX-2-related PGE_2 production may play a prominent role in mediating the proangiogenic effect of histamine in human colorectal cancer.

In conclusion, we showed that up-regulation of histidine decarboxylase activity was correlated with tumor progression (i.e., the presence of lymph node and/or distant metastases) in human colorectal cancer. The present study is the first to show a novel role of histamine in activating the COX-2 pathway via H_2 and H_4 receptors in COX-2-expressing colon cancer cells. It is likely that COX-2-related PGE_2 production acts as one of the most important mediators of the proproliferative and proangiogenic effects of histamine in colorectal tumors. Collectively, these data point to the dual inhibition of the histidine decarboxylase and COX-2 pathways as a possible therapeutic tool in the treatment of colorectal cancer.

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