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ORIGINAL ARTICLE

Mast cells have a protumorigenic role in human thyroid cancer

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In different human carcinoma types, mast cell infiltrate increases with respect to normal tissue and mast cell density correlates with a bad prognosis. To assess the role of mast cells in human thyroid cancer, we compared the density of tryptase-positive mast cells in 96 papillary thyroid carcinomas (PTCs) versus normal thyroid tissue from 14 healthy individuals. Mast cell density was higher in 95% of PTCs ($n = 91$) than in control tissue. Mast cell infiltrate correlated with extrathyroidal extension ($P = 0.0005$) of PTCs. We show that thyroid cancer cell-line-derived soluble factors induce mast cell activation and chemoattraction *in vitro*. Different mast cell lines (HMC-1 and LAD2) and primary human lung mast cells induced thyroid cancer cell invasive ability, survival and DNA synthesis *in vitro*. The latter effect was mainly mediated by three mast-cell-derived mediators: histamine, and chemokines CXCL1/GROα and CXCL10/IP10. We show that xenografts of thyroid carcinoma cells (8505-C) could recruit mast cells injected into the tail vein of mice. Co-injection of human mast cells accelerated the growth of thyroid cancer cell (8505-C) xenografts in athymic mice. This effect was mediated by increased tumor vascularization and proliferation, and was reverted by treating mice with sodium cromoglycate (Cromolyn), a specific mast cell inhibitor. In conclusion, our study data suggest that mast cells are recruited into thyroid carcinomas and promote proliferation, survival and invasive ability of cancer cells, thereby contributing to thyroid carcinoma growth and invasiveness.
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Introduction

Inflammation was first linked to cancer in the nineteenth century consequent to observations that tumors often arose at sites of chronic inflammation, and that inflammatory cells were present around tumors. Several chronic inflammatory diseases predispose to cancer. Cytokines, secreted by stromal cells, can exacerbate the malignant phenotype of cancer cells. These, by producing chemoattractant molecules, recruit inflammatory cells into tumor sites, influencing them in a way that ultimately promotes cancer progression (Coussens and Werb, 2002; Balkwill, 2004; Mantovani *et al*., 2008).

There are four types of thyroid carcinomas: differentiated papillary thyroid carcinomas (PTCs), follicular carcinomas, poorly differentiated carcinomas and undifferentiated carcinomas. PTC is the most common thyroid malignancy and it is usually associated with a good therapeutic response and prognosis. However, 10% of PTC patients develop recurrences and distant metastases. At the somatic level, PTC is associated with four genetic lesions, namely, chromosomal aberrations affecting the RET or TRKA tyrosine kinase receptors and point mutations in the *RAS* or *BRAF* genes (Kondo *et al*., 2006).

Inflammatory infiltrates have been found in human thyroid tumors. An association between PTC and lymphocytic thyroiditis has been reported in various studies since the 1950s (Di Pasquale *et al*., 2001; Rhoden *et al*., 2006). In addition, macrophages and immature dendritic cells accumulate in PTCs, both in tumoral stroma and at the invasive front (Scarpino *et al*., 2000; Ryder *et al*., 2008).

Mast cells are bone-marrow-derived immune cells widely distributed throughout vascularized tissues, and at interfaces with the external environment (Marone *et al*., 2005; Kalesnikoff and Galli, 2008). Mast cells are present in several tumors where they influence many aspects of tumor biology (Coussens *et al*., 1999; Nakayama *et al*., 2004; Soucek *et al*, 2007; Huang *et al*., 2008; Yang *et al*., 2008) such as angiogenesis (Detoraki *et al*., 2009) and tumor invasion of the extracellular matrix by releasing cytokines and proteases (Coussens *et al*., 1999). The role of mast cells in tumor growth is supported by the observation that carcinoma formation and expansion is greatly reduced in mast-cell-deficient KitW-sh/W-sh mutant mice (Coussens *et al*., 1999; Soucek *et al*, 2007).

In this study, we investigated the possibility that mast cells and their mediators have a role in human thyroid cancer. We found that human thyroid carcinomas feature a significant mast cell infiltrate whose intensity

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correlates with the invasive phenotype. Using chemoattraction assays, we show that thyroid cancer cells attract mast cells through the release of vascular endothelial growth factor-A (VEGF-A). The administration of HMC-1 mast cells into the tail vein of nude mice caused their recruitment to thyroid-carcinoma-cell-induced xenografts. Thyroid cancer cells stimulate histamine release and cytokine synthesis in human mast cells. Mast-cell-released mediators enhanced the proliferation, survival and invasive behavior of thyroid cancer cells *in vitro*. Moreover, mast cells promote the growth of thyroid carcinoma xenografts in nude mice, and this effect could be blocked by sodium cromoglycate (Cromolyn), a specific mast cell inhibitor. Our data indicate that mast-cell-derived chemokines and histamine are the major mediators of the growth-inducing effect of mast cells on thyroid cancer cells.

Results

Mast cell density is increased in human thyroid cancer and correlates with invasiveness

Samples from normal and pathological thyroid tissues were evaluated by immunohistochemistry for mast cell infiltrate. We evaluated the density of tryptase-positive cells in PTCs ($n=96$) and in thyroid tissues from healthy individuals ($n=14$) whose average age and gender were comparable to PTC samples (Supplementary Table 1). Mast cells were counted in each sample and cases were classified depending on the mast cell number, as indicated in the Materials and methods section. Mast cell infiltrate was present both within the tumors and at the invasive front, but was virtually absent from normal tissues: of the 14 healthy thyroid samples, 13 were negative for mast cells and the remaining 1 was weakly (+) positive for tryptase. Instead, mast cell density was increased, to various degrees, in 95% of PTCs ($n=91$). Thus, the presence of mast cell infiltrate distinguished normal tissues from carcinomas ($P=0.0001$). A representative example of cell staining with tryptase antibody is shown in Figure 1. An isotype-matched non-related antibody gave negative results for both normal and pathological samples (not shown).

We then looked for correlations between clinicopathological features (Supplementary Table 1), angiogenesis and mast cell infiltrate. To this aim, PTCs were divided into two groups, those with intense (+++, ++) tryptase staining, which represented 65% (63 of 96) of the samples and those with weak (−, +) tryptase staining, which represented 35% (33 of 96) of the samples. The group of PTCs with intense tryptase staining tended to be more invasive, that is, extrathyroidal extension was more frequent ($P=0.0005$; Table 1). Tryptase staining did not correlate with gender, age, PTC histological variant, tumor size, lymph node metastasis, multifocality or the degree of angiogenesis (data not shown). These results indicate that mast cells are present in PTCs, but not in normal thyroid tissues, and that their presence and intensity positively correlate with invasive behavior.

Recruitment of mast cells by human thyroid carcinoma cells: a role for VEGF-A

To evaluate whether the increased number of mast cells in a tumor site could be because of mast cell recruitment by thyroid carcinoma cells, we performed *in vitro* chemotaxis assays. For these experiments, we used two human mast cell lines, HMC-1 and LAD2, derived, respectively, from a patient with mast cell leukemia and a patient with mastocytosis (Butterfield *et al*., 1988; Kirshenbaum *et al*., 2003). We tested the ability of conditioned culture media (CM) from the thyroid carcinoma cell lines TPC1, NIM and 8505-C to induce migration of mast cells through a fibronectin matrix. Figure 2a shows that CM from each of the three thyroid carcinoma cell lines induced migration of both LAD2 and HMC-1 cells; nonconditioned CM was used as a negative control. CM from normal thyroid primary cultures did not induce mast cell migration (not shown). These data indicate that soluble factors secreted by thyroid cancer cells were responsible for the chemoattractant effect exerted on mast cells.

A likely candidate for the chemoattractant effect is VEGF-A, as we and others demonstrated that thyroid carcinoma cells produce VEGF-A (Viglietto *et al*., 1995; de la Torre *et al*., 2006), and human mast cells and basophils express the VEGF-A receptors VEGFR-1 and -2 (de Paulis *et al*., 2006; Detoraki *et al*., 2009). As shown in Figure 2b, thyroid carcinoma, but not normal cells produced high levels of VEGF-A. We then blocked the activity of this cytokine in CM from TPC1 cells with three neutralizing VEGF-A antibodies and found that mast cell chemotaxis was significantly impaired (Figure 2c). These data indicate that human thyroid cancer cells secrete VEGF-A, and that this cytokine functions as chemoattractant for mast cells.

Human thyroid carcinoma cells induce mast cell activation

Human mast cells release a large array of mediators (Kalesnikoff and Galli, 2008), including histamine. We stimulated LAD2 cells with thyroid carcinoma cell CM, and evaluated histamine release by ELISA assay. Figure 3a shows that both TPC1 and 8505-C CM concentration dependently stimulated histamine release from LAD2, TPC1 CM being almost as powerful as the Ca2+ ionophore A23187. Histamine concentrations in 8505-C CM and TPC1 CM were undetectable (not shown). Similar results were obtained when HMC-1 were activated by both TPC1 CM and 8505-C CM (Figure 3b). Significantly, when purified mast cells from human lung mast cells (HLMCs) were treated with TPC1 CM and 8505-C CM, they reproducibly underwent degranulation, as assessed by the measure of histamine release. A representative experiment is shown in Figure 3c.

We verified whether thyroid carcinoma cell CM could also induce a transcriptional response in mast cells. Figure 4a shows that 24 h treatment of mast cells with thyroid carcinoma cell CM significantly upregulated the mRNA of cytokines (interleukin-6, granulocyte-macrophage colony-stimulating factor and tumor necrosis

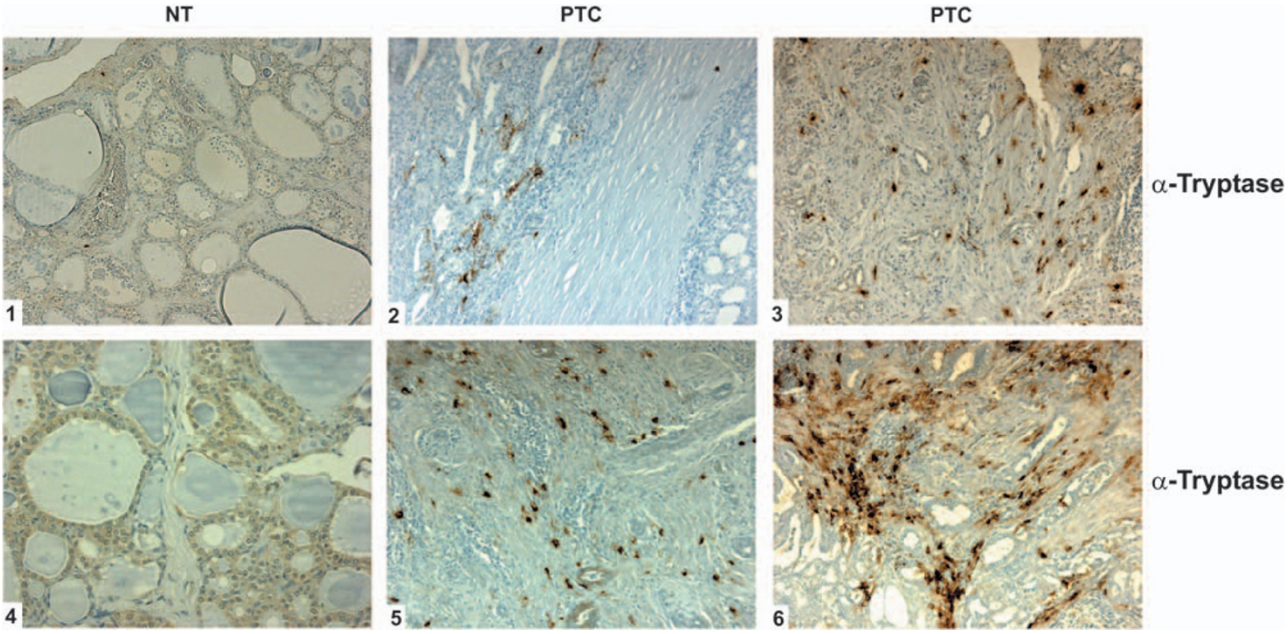


Figure 1 Immunohistochemical analysis of tryptase in human papillary thyroid carcinoma (PTC). Normal thyroid tissues (1, 4) were negative for tryptase staining. The degree of tryptase-positive cell staining differed among PTC samples (see samples, 2, 3, 5 and 6). No signal was detected after incubation of samples with isotype-matched antibodies (not shown).

Table 1 Correlations between tryptase score and clinical features of papillary thyroid carcinomas

Tryptase score	*Clinical features*		P-*value*[a]
	Extrathyroidal extension −	*Extrathyroidal extension +*	
Intense tryptase staining	45% (17/38)	77% (46/58)	**0.0005**
Weak tryptase staining	55% (21/38)	23% (12/58)	

[a]The χ^2-test was used to establish the statistical significance of distributions. Mast cell infiltrate correlated with capsule invasion and bilateral occurrence.
The *P*-value, represented in bold, indicates 'extremely significant'.

factor-α) and chemokines (CXCL1/Groα and CXCL10/IP10). As shown in Figure 4b, CXCL1 was abundantly secreted by HMC-1 and LAD2 cells stimulated with thyroid carcinoma cell CM. The pattern of induced cytokines was different for each CM. For instance, TPC1 CM was more efficient than NIM CM in inducing CXCL10 mRNA. This indicates that a complex mixture of mediators, contained in the different CM, is responsible for mast cell activation. These mediators are still to be identified.

Mast-cell-derived mediators induce proliferation, survival and invasive ability of thyroid carcinoma cells

We measured bromodeoxyuridine (BrdU) incorporation, as an indication of DNA synthesis, after 48 h of treatment of thyroid carcinoma cells (TPC1 and 8505-C) with HMC-1 and LAD2 CM. Figure 5a shows that HMC-1 and LAD2 CM increased BrdU incorporation in thyroid carcinoma cells compared with the non-conditioned medium. P5 normal thyroid primary cultures were not affected by treatment with HMC-1 and LAD2 CM.

To exclude the possibility that the observed proproliferative effect was because of the neoplastic phenotype of the mast cell lines, we performed the same experiment with HLMC. In the presence of HLMC CM, there was an increase in BrdU incorporation of TPC1 and 8505-C cells comparable to that induced by HMC-1 and LAD2 (Figure 5b). To ascertain that this effect was specific of mast cells, and not a common feature of human bone-marrow-derived cells, we used peripheral blood lymphocytes (PBL) from different donors, and the Jurkat cell line, which is derived from a human T-cell leukemia. Conditioned media of PBL and Jurkat cell lines were significantly less efficient in inducing proliferation of TPC1, and did not induce any proliferation of 8505-C cells. Purified monocytes, obtained from peripheral blood mononuclear cells and selected through cellular adhesion, did not show a significant effect on 8505-C cell proliferation (data not shown). Indeed, BrdU incorporation increased from 10 to 30% in the presence of HLMC CM, but not in the presence of PBL or Jurkat CM (Figure 5b). We then evaluated whether mast cell CM inhibited apoptosis of thyroid carcinoma cells. To this aim, TPC1 and 8505-C cells were serum-deprived for 48 h, a condition that induces apoptosis. Figure 5c shows that both HMC-1 and LAD2 CM strongly reduced the levels of serum starvation-induced apoptosis. Thus, soluble factor(s) secreted by mast cells are able to stimulate S-phase entry and inhibit apoptosis of thyroid carcinoma cells.

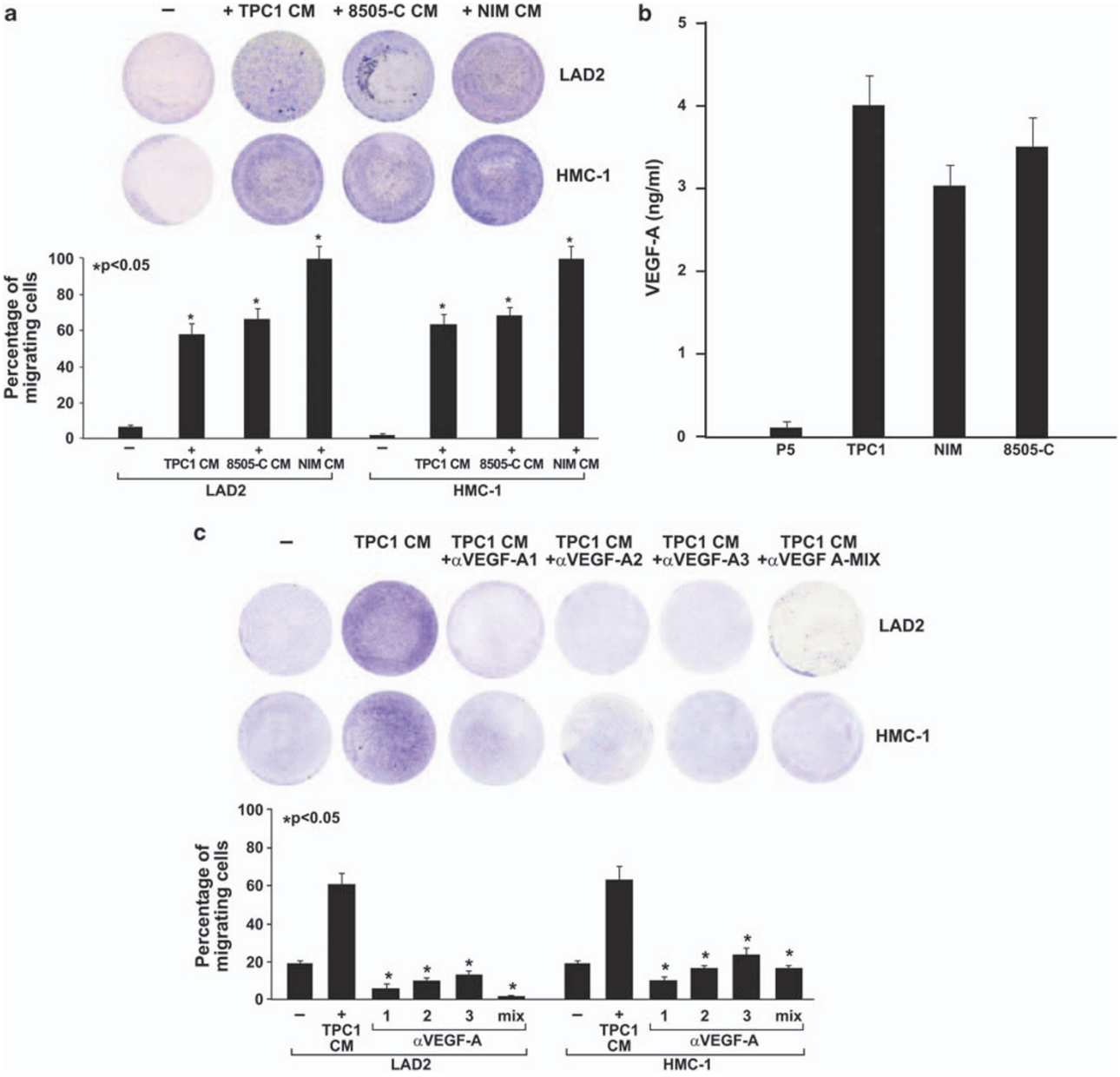


Figure 2 Migration of LAD2 and HMC-1 cells in response to thyroid cancer cell line-conditioned media (CM). (**a**) Mast cells were plated on the top of a transwell chamber precoated with fibronectin and incubated with thyroid carcinoma cell CM (TPC1, 8505-C, NIM CM) or nonconditioned medium (−). Migrated cells were fixed and stained with crystal violet, and the percentage of migration was evaluated with an ELISA reader, and calculated with respect to total cell number (1×10^5). Top, representative micrographs. Bottom, average results of three independent assays. (**b**) VEGF-A expression in human thyroid cell lines was evaluated by ELISA. The TPC1, NIM and 8505-C cell lines released high levels of VEGF-A with respect to the normal primary thyroid P5 cells, as assessed in triplicate determinations (±s.d.). (**c**) Matrigel invasion of mast cells in response to TPC1 CM was performed in the presence and absence of three distinct anti-VEGF-A blocking antibodies. Each antibody, and the mix of them, inhibited mast cell migration. Top, representative micrographs. Bottom, average results of three independent assays.

We evaluated whether mast cells could directly influence the invasive behavior of thyroid carcinoma cells through Matrigel chemoinvasion assays. Thyroid carcinoma cells were seeded on the top chamber of transwells, and their ability to invade a reconstituted extracellular matrix (Matrigel) toward mast cell CM was evaluated. Mast cell CM, but not nonconditioned media, induced thyroid carcinoma cell migration in Matrigel (Figure 5d).

Mast-cell-derived mediators involved in thyroid cancer cell proliferation

We next asked whether histamine could mediate mast-cell-induced proliferation of thyroid carcinoma cells. By using qRT–PCR, we found that H_1 and H_2 receptors were markedly upregulated in thyroid carcinoma cell lines. Normal thyroid cells were negative for each of the histamine receptors (Supplementary Figure 1A). These data were confirmed by western blot analysis

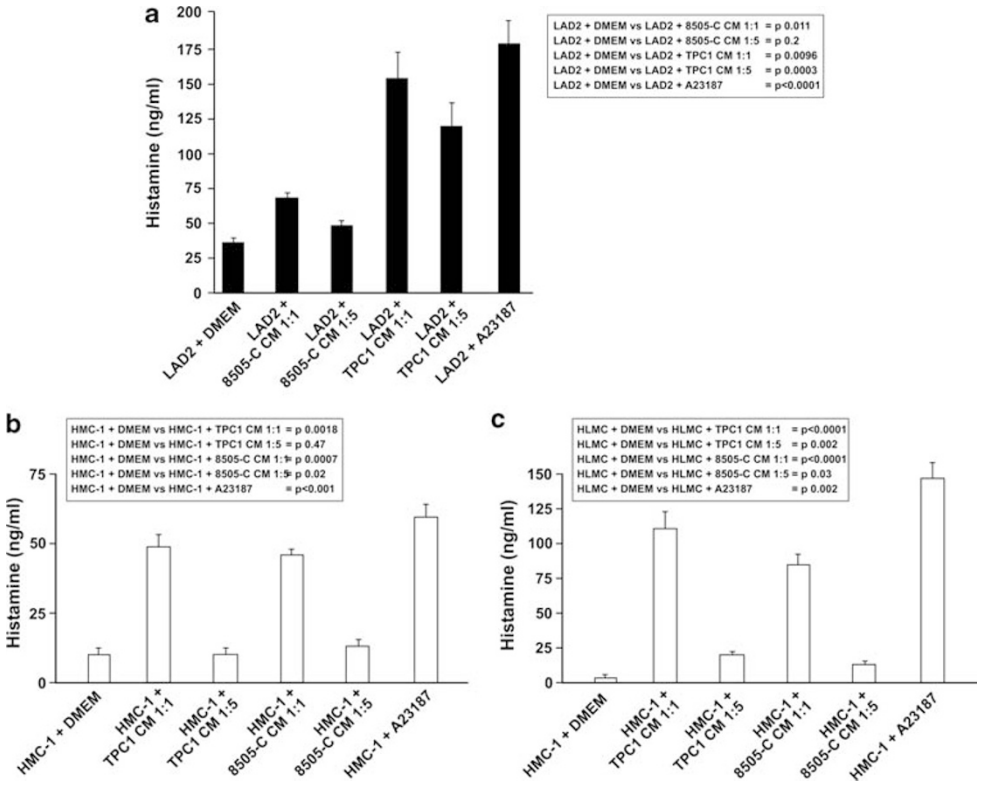


Figure 3 Histamine release of human mast cells in response to thyroid carcinoma cell CM. (**a**) LAD2 cells were incubated 1 h at 37 °C with the indicated dilutions of 8505-C CM, TPC1 CM or with the Ca^{2++} ionophore A23187 (1 μg/ml). The release of histamine in the supernatants of mast cells was evaluated by ELISA. Both 8505-C CM and TPC1 CM stimulated histamine release of mast cells. Dilutions (1:5) of both thyroid cancer cell CM were obtained with non-conditioned DMEM and abrogated this effect. Histamine release was not detected in the presence of non-conditioned medium. (**b**) HMC-1 was incubated with 8505-C CM, TPC1 CM or with the Ca^{2+} ionophore A23187. Both 8505-C CM and TPC1 CM stimulated histamine release of HMC-1 cells. (**c**) Histamine release was also evaluated in HLMC. On incubation with 8505-C CM, TPC1 CM or with the Ca^{2+} ionophore A23187, HLMC cells released histamine.

(Supplementary Figure 1B). Then, we evaluated the ability of histamine to enhance the S-phase entry rate of thyroid carcinoma cell lines. Consistent with the finding that thyroid carcinoma cells express H_1 and H_2 receptors, histamine (10^{-7} to 10^{-4} M) concentration dependently enhanced S-phase entry in TPC1 and 8505-C cells. Moreover, the specific inhibitors of receptors H_1 (levocitirizine) and H_2 (ranitidine) blocked this effect (Supplementary Figure 2). Histamine (10^{-5} M) induced an increase of S-phase entry (Figure 6a).

Because histamine could not account for the total DNA synthesis-stimulating activity of mast cell CM, we hypothesized that other mediators might be involved. We previously demonstrated that two chemokines, CXCL1/GROα and CXCL10/IP10, were important mediators of PTC cell proliferation and invasive ability (Melillo *et al*., 2005). Moreover, in this study, thyroid carcinoma CM induced upregulation of the expression and secretion of these chemokines in HMC-1 and LAD2 cells (Figure 4). Consequently, we evaluated whether CXCL1 and CXCL10 could be involved in the proliferative effect caused by mast cell CM, and found that both the chemokines induced BrdU incorporation of TPC1 and 8505-C cells with a higher efficiency than histamine (Figure 6a). Moreover, histamine enhanced CXCL1- and CXCXL10-induced proliferation. When all three mediators were added to the medium together, there was a fivefold increase in BrdU incorporation (Figure 6a). To verify these results, we used CXCL1- and CXL10-neutralizing antibodies to immunodeplete mast cell CM of the two ligands. Chemokine immunodepletion was confirmed by ELISA assay (not shown). The removal of each chemokine from mast cell CM caused the proliferation rate of TPC1 cells to return to basal level. The addition of the recombinant chemokines to the immunodepleted mast cell CM rescued the proliferation effect of the complete medium (Figure 6b). These data suggest that CXCL1 and CXCL10 are the major mediators of the mast cell CM-induced proliferation of thyroid carcinoma cells identified in this study.

Xenografts of human thyroid cancer cells can recruit circulating mast cells

To show that human mast cells can be recruited into tumor site *in vivo* and enhance cancer cell proliferation, we injected nude mice with 8505-C (10×10^6) thyroid carcinoma cells. At 5 weeks, tumors measured approximately 40 mm^3, and HMC-1 cells (1:10) were injected

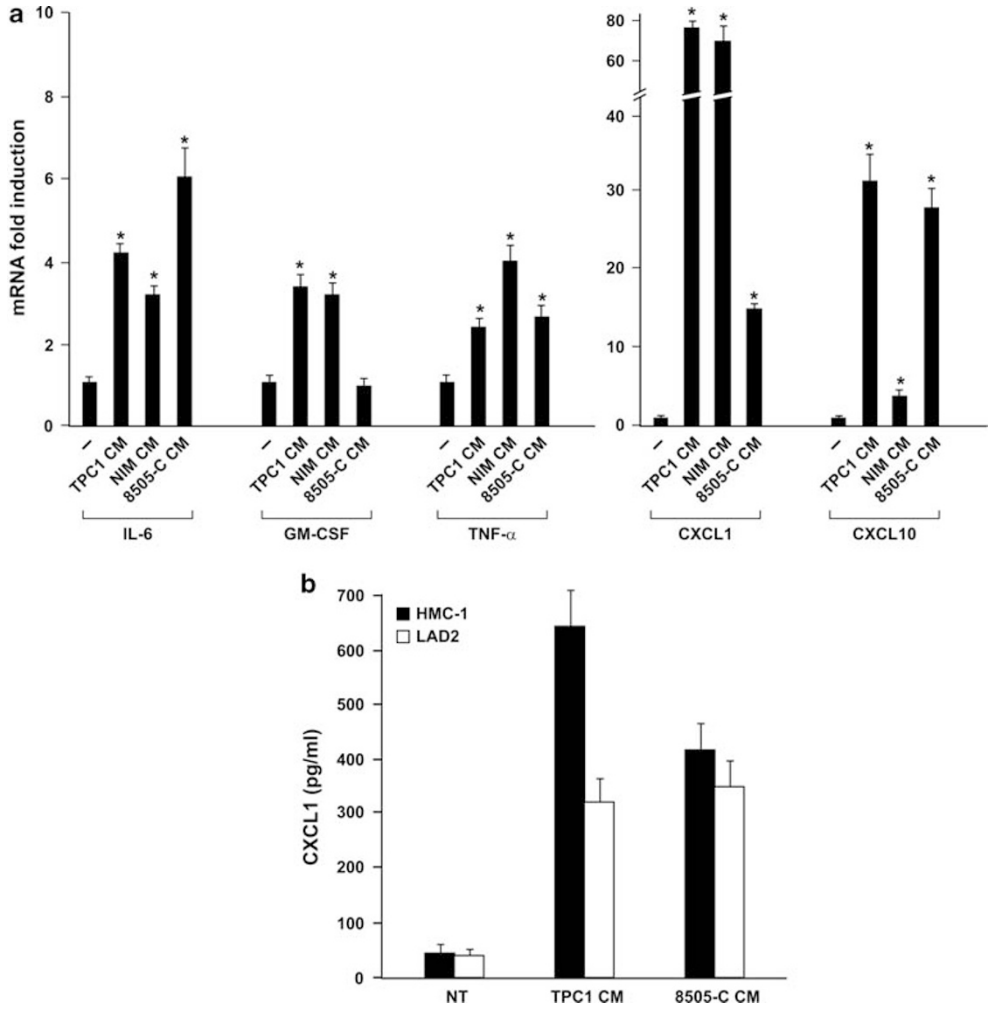


Figure 4 Activation of HMC-1 in response to thyroid carcinoma cell CM. (**a**) Quantitative RT–PCR was used to evaluate the mRNA levels of cytokines and chemokines of HMC-1 cells in response to the indicated thyroid carcinoma cell line CM. Nonconditioned medium (−) was used as a control. β-Actin mRNA detection was used for normalization. For each target (*x* axis), the expression level values are calculated relative to the expression level in unstimulated HMC-1 cells, arbitrarily considered equal to 1. Experiments were performed in triplicate, and the average value of the results ± s.d. was plotted. $*P<0.05$. (**b**) CXCL1 expression in human mast cell lines was evaluated by ELISA. Both HMC-1 and LAD2 mast cell lines released high levels of CXCL1 in response to the indicated thyroid carcinoma cell line CM, as assessed in triplicate determinations (± s.d.) of CXCL1. Normal thyroid cell (NT) conditioned medium was used as negative control.

into the tail vein of mice. Animals were then killed at 1, 2 and 7 days after injection, and excised tumors were subjected to staining with anti-tryptase and anti-ki67 antibodies. Rare scattered clusters of mast cells were observed almost exclusively at tumor edges of HMC-1-injected mice (8505-C and HMC-1), but not in noninjected controls. An example of mast cell clusters in xenografts (day 7) is shown in Figure 7b. Very few mast cells were counted at day 1 (approximately 5–10 in the whole tumor section), but their number increased at day 7 after injection (Figure 7a). The percentage of $ki67^+$ cells also increased in xenografts of HMC-1-injected mice, but not in controls (Figure 7a). Tryptase-positive cells were negative for ki67. These data indicate that, at this time point, mast cells do not proliferate, but rather induce the proliferation of thyroid cancer cells in the tumor site.

Mast cells enhance the xenograft growth of human thyroid cancer cells in athymic nu/nu mice

The experiments described above suggested that mast cells can be recruited into tumor site by thyroid cancer cells and enhance tumor growth. To extend this observation, we injected three groups of 10 mice each, respectively, with 10×10^6 8505-C cells, 1×10^6 HMC-1 cells, and a mixture of both cells. As shown in Figure 8a, HMC-1 cells alone did not induce tumor formation. In contrast, 8505-C cell xenografts formed tumors at 4 weeks after injection, which progressively increased. When 8505-C cells were co-injected with HMC-1 cells, tumors occurred earlier, and the final tumor volume was consistently higher than those of 8505-C tumors ($P<0.001$). Similar results were obtained with LAD2 cells (Supplementary Figure 3).

End-stage tumors excised from the animals at 6 weeks after inoculation were immunostained for tryptase,

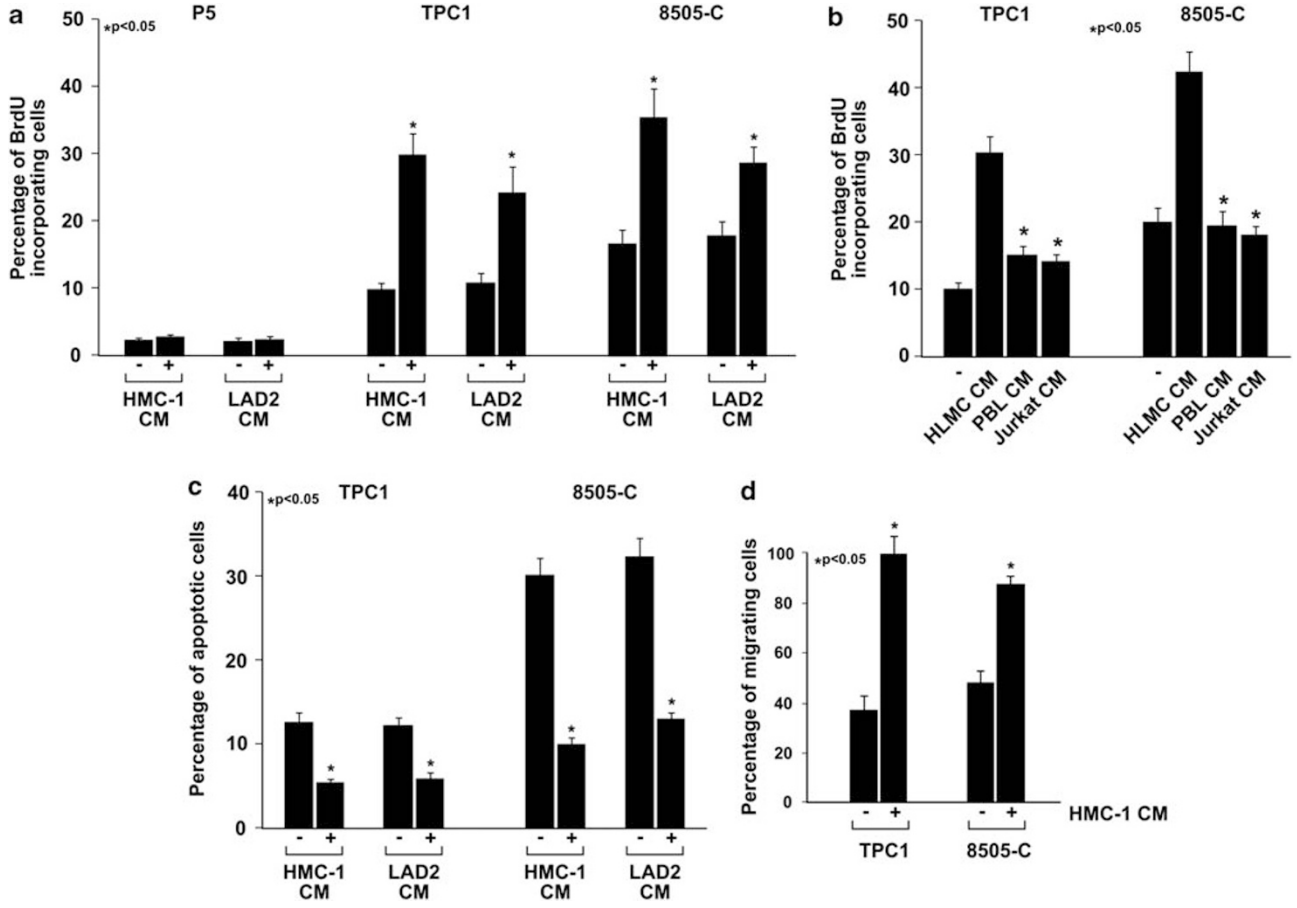


Figure 5 Mast cells stimulate malignant features of thyroid carcinoma cell lines. (**a**) The indicated thyroid carcinoma cell lines were serum deprived for 12 h and stimulated with mast cell conditioned culture medium (CM) or with nonconditioned medium for 48 h. BrdU was added 2 h before the reaction was stopped, and cells were processed for immunofluorescence. Mast cell CM stimulated S-phase entry of thyroid carcinoma cell lines, but not of the P5 normal primary thyroid cultures. The results are expressed as percentage of BrdU incorporation with respect to unstimulated cells. (**b**) TPC1 and 8505-C cells were serum deprived and then stimulated with CM from human primary lung mast cells (HLMC), peripheral blood lymphocytes (PBL) and Jurkat T-cell leukemia cell line. Mast cell CM, but not CM from the other cultures, induced BrdU incorporation of thyroid cancer cells. (**c**) The indicated thyroid carcinoma cell lines were serum deprived for 48 h in the presence of mast cell CM or nonconditioned medium. The percentage of apoptotic cells was evaluated with the TUNEL reaction. Mast cell CM, but not nonconditioned medium, induced survival of TPC1 and 8505-C cells in condition of serum starvation. The average results and s.d. of three independent experiments are reported. (**d**) The indicated thyroid carcinoma cell lines were seeded in the upper chamber of Matrigel-coated 8 μM pore transwells and allowed to migrate for 24 h toward HMC-1 CM or nonconditioned medium. The average results of three independent assays and s.d. are shown.

CD34 and ki67, markers for mast cells, endothelial cells and cycling cells, respectively. Immunostaining for tryptase (Figure 8b) revealed HMC-1 cells in mixed xenografts. Evaluation of xenografts showed that tryptase-positive cells represented 10–20% of all cells, indicating that mast cells survive and proliferate in the presence of thyroid carcinoma cells. Immunostaining for ki67 (Figure 8b) showed that mixed xenografts had a higher proliferative index than tumors induced by 8505-C cells alone. In the latter, 38% of cells were ki67$^+$ versus 68% in mixed xenografts. We also evaluated vessel number and diameter in tumor tissues using the CD34 antibody. This analysis revealed a significant enhancement of vascularization in 8505-C/HMC-1 tumors compared with 8505-C tumors (Figure 8b). To ascertain the identity of proliferating (ki67$^+$) cells in xenografts, we generated an 8505-C cell population expressing the green fluorescent protein (8505/GFP). These cells formed tumors with the same efficiency as that of parental cells. The co-staining of xenografts for ki67 and GFP showed that the majority of the proliferating cells were GFP$^+$. Moreover, 45% of the GFP$^+$ cells were proliferating in 8505-C/GFP xenografts, whereas 74% of the GFP$^+$ cells were proliferating in 8505-C/GFP + HMC-1 xenografts (Figure 8b). These data indicate that mast cells favor the proliferation of thyroid cancer cells into the tumor site. To demostrate that mast cell blockade could inhibit tumor growth in our model system, we used a mast cell inhibitor, disodium chromoglycate (Cromolyn). 8505-C and 8505-C/HMC-1 xenografts were induced and, when they reached the size of 40 mm^3, Cromolyn or vehicle was administered daily by intraperitonal injection for 5 days a week. As shown in Figure 8c, 8505-C xenografts were not affected by Cromolyn treatment, whereas mixed xenografts were strongly inhibited. At 2 weeks of

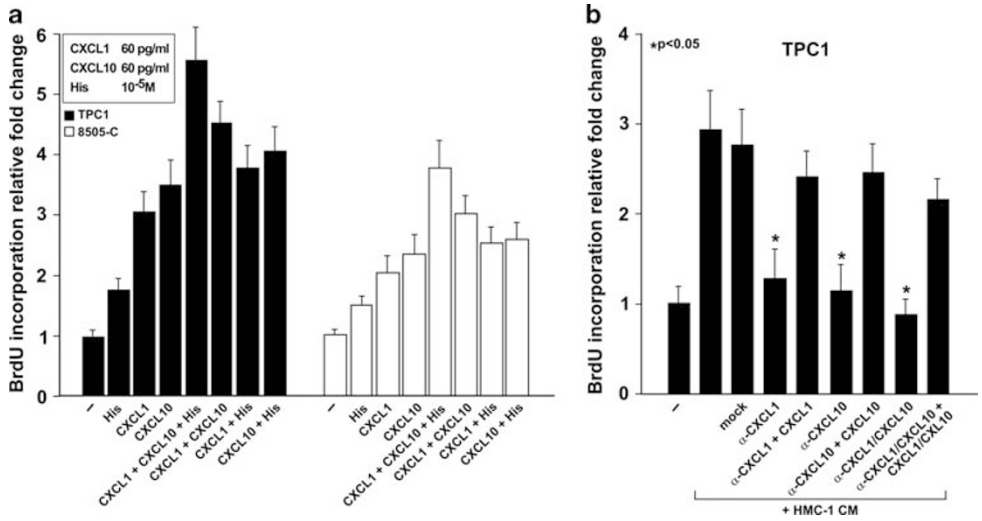


Figure 6 The chemokines CXCL1 and CXCL10 and histamine mediate the proliferative effect of mast cell CM on thyroid carcinoma cell lines. (**a**) BrdU-incorporation was measured to evaluate S-phase entry of TPC1 and 8505-C cells on treatment with CXCL1 (60 pg/ml), CXCL10 (60 pg/ml), histamine (10^{-5} M) or the indicated combinations of these factors. As shown, histamine potentiated the effect of each chemokine. BrdU incorporation was highest when the three molecules were added together. (**b**) Neutralizing antibodies to CXCL1 and CXCL10 were used to deplete mast cell CM from chemokines. Immunodepletion was confirmed by ELISA assay (not shown). Chemokine-immunodepletion abrogated the proliferative effect of mast cell CM on TPC1 cells, whereas mock immunodepletion (performed with isotype-matched unrelated antibodies) did not. The add back of excess chemokine to immunodepleted media restored the effect of mast cell CM on TPC1 cells proliferation. The average results of three independent experiments ± s.d. are shown.

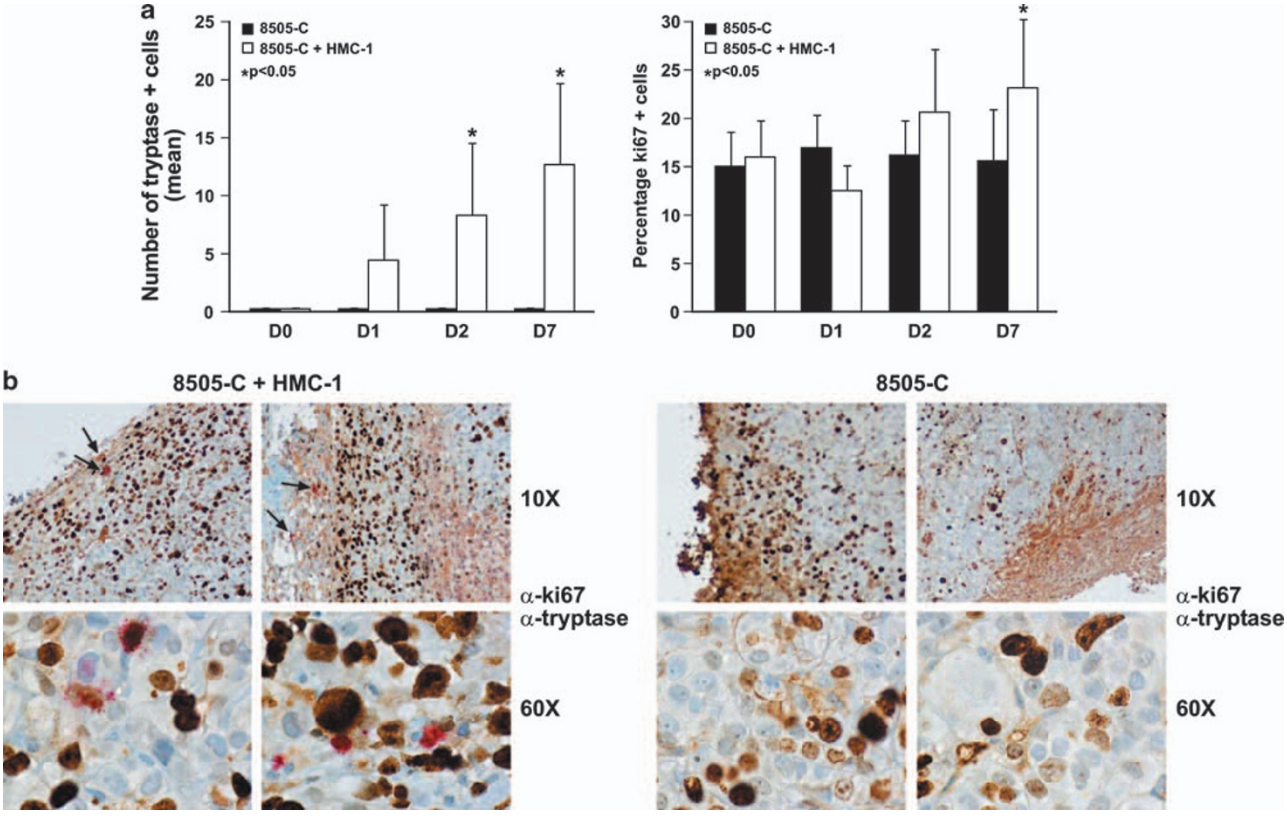


Figure 7 Recruitment of mast cells by thyroid carcinoma xenografts in nude mice. (**a**) HMC-1 cells were injected into the tail vein of nude mice bearing 8505-C xenografts. Animals were killed at the indicated time points and tryptase$^+$ and ki67$^+$ cells were counted in whole tumor sections. The mean number of tryptase$^+$ and ki67$^+$ cells, reported in the bar graph shown above, was obtained by evaluating at least three sections for each tumor. For each group of mice (8505-C and 8505-C + HMC-1), at least 10 tumors were evaluated. (**b**) Immunohistochemical analysis of 8505-C + HMC-1 xenografts at day 7 shows selected areas of tumor borders were scattered clusters of mast cells (indicated by arrows) are present. In the lower panel, a magnification of these areas is shown. Tryptase and ki67 staining is shown, respectively, in red and in brown. 8505-C xenografts are completely negative for tryptase.

treatment, vehicle-treated 8505-C/HMC-1 mean tumor size was 1200 mm^3, whereas Cromolyn-treated 8505-C/HMC-1 mean tumor size was 420 mm^3. Cromolyn treatment, although effective in inhibiting tumor growth, did not cause the growth of mixed xenografts to return to the levels of 8505-C xenografts. Ki67 immunohistochemistry showed decreased proliferation of Cromolyn-treated tumors with respect to control xenografts (Figure 8d). Co-labeling of tumor specimens with anti-ki67 and anti-tryptase antibodies revealed that mast cell proliferation was not affected by Cromolyn treatment. Cromolyn did not affect HMC-1 nor 8505-C cell growth *in vitro* (data not shown). Thus, the inhibition of mast cell function blocked tumor expansion by impeding proliferation of cancer cells.

Discussion

Mast cells are present in several tumors, in which they can mediate protumorigenic or antitumorigenic activities (Theoharides and Conti, 2004; Welsh *et al*., 2005). Chronic inflammatory reactions occur in human PTCs and in genetically modified mice models predisposed to develop thyroid carcinomas (Rhoden *et al*., 2006; Pufnock and Rothstein, 2009), and an inflammatory transcriptional response can be induced in normal thyroid epithelial cells on expression of PTC oncogenes. This response involves chemokines and VEGF-A (Borrello *et al*., 2008). VEGF-A was identified also in human thyroid cancer samples, where it correlated with tumor aggressiveness (Klein *et al*., 2001; Jo *et al*., 2006), and it has been linked to activation of BRAF–ERK signaling also in melanomas (Sumimoto *et al*., 2006). Interestingly, VEGF-A induces human mast cell chemotaxis by activating VEGFR1 and VEGFR2 (Detoraki *et al*., 2009). We found that mast cell density is much greater in human thyroid cancer tissue than in normal thyroid tissue. This observation may be because of: (1) recruitment of mast cell precursors or (2) increased proliferation/survival of resident mast cells. We show, by using chemoattraction assays, that thyroid carcinoma cell cultures are a potent chemoattractant for mast cells. This effect required thyroid carcinoma-cell-derived VEGF-A because it was inhibited by an anti-VEGF-A antibody. Consistently, mast cells (HMC-1) injected in the tail vein of immunodeficient mice are recruited to thyroid carcinoma cell (8505-C) xenografts. Interestingly, when 8505-C and HMC-1 cells were co-injected subcutaneously, mast cells survived and proliferated. This finding indicates that thyroid cancer cells provide mast cells with a favorable environment that ultimately promotes their survival and growth. Consequently, the greater cell density in human thyroid cancer tissue could be because of both recruitment of mast cell precursors and increased proliferation/survival of resident mast cells.

We also show that thyroid carcinoma cell CM induces mast cell activation (that is, histamine release and cytokine synthesis). Human mast cells can be activated by immunological and nonimmunological stimuli through distinct signaling pathways (Gilfillan and Tzaczyc, 2006). How mast cells are activated in our model system is not known, and will be the aim of future investigation.

We previously showed that thyroid carcinoma cells express CXCR2 and CXCR3, and their ligands, CXCL1 and CXCL10. These autocrinously produced chemokines sustain PTC cell proliferation and invasion (Melillo *et al*., 2005). In the same study, the addition of exogenous CXCL1 and CXCL10 to PTC cells further enhanced their proliferation and Matrigel invasion, which suggests that the CXCR2 and CXCR3 receptors on PTC cells remain available for exogenous ligand binding. The results of this study suggest that the mast-cell-derived chemokines CXCL1 and CXCL10 are probably the mediators responsible for the mast cell CM-induced growth-inducing effect on thyroid carcinoma cells. These findings support the hypothesis that mast-cell-derived CXCL1 and CXCL10 might be one of the sources of exogenous chemokines at tumor sites.

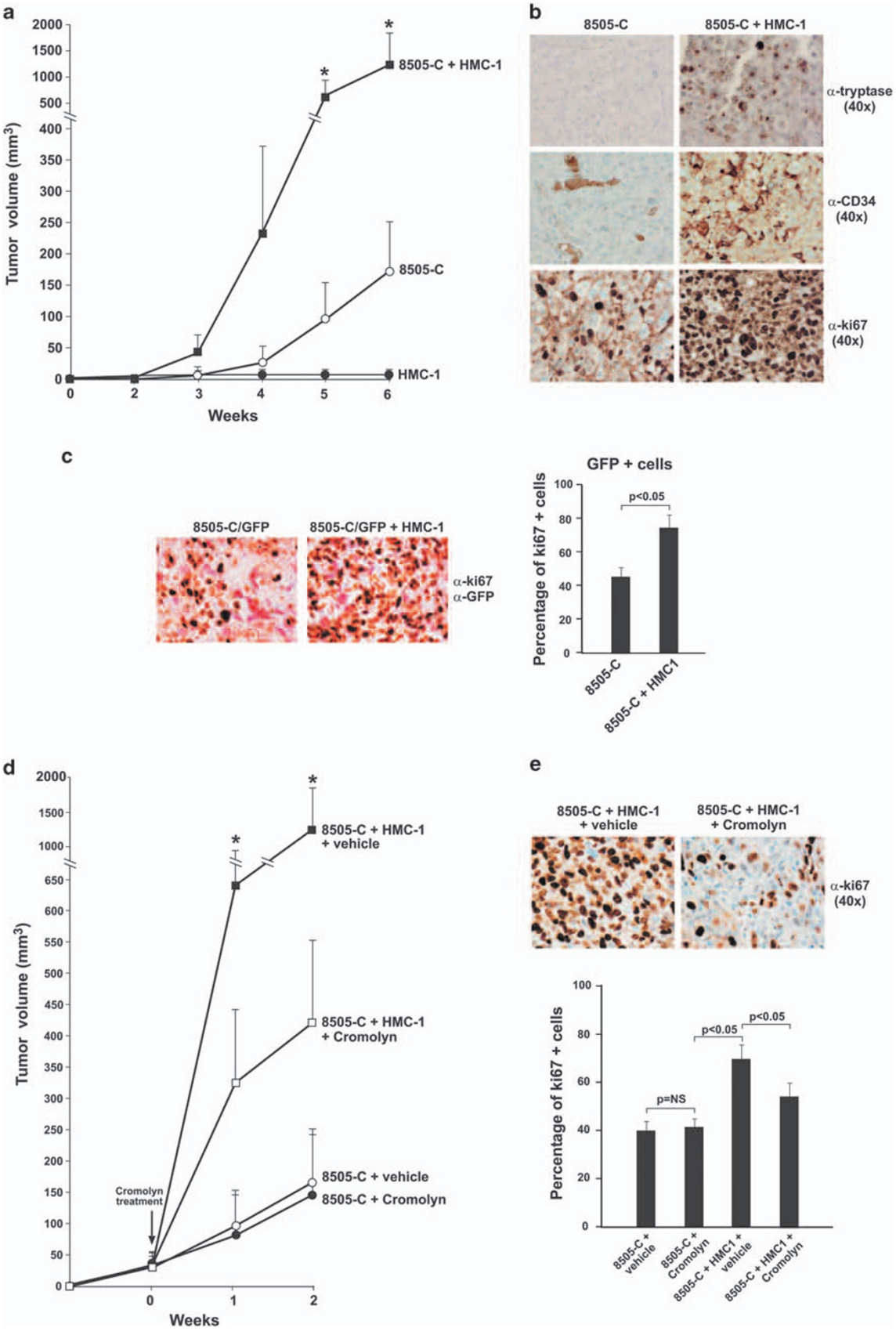
In this study, we report that histamine contributes to the growth-inducing activity of mast cell CM on thyroid carcinoma cells. Although still controversial, several studies identified H_1 and/or H_2 receptor expression in cancer cells and showed that histamine functions as a growth factor (Rivera *et al*., 2000). In our system, histamine exerts an autonomous effect on cell growth and potentiates chemokine-induced cell proliferation. This effect appears to be mediated by the H1 and H2 receptors expressed by thyroid carcinoma cells, as specific inhibitors of H_1 and H_2 reverted histamine effects. Both CXC and histamine receptors are G-protein-linked receptors (Parsons and Ganellin, 2006; Tan *et al*., 2006; Raman *et al*., 2007). Whether these receptors show typical coupling to signal transducers and how signaling pathways associated with these receptors integrate and induce cell proliferation will be further investigated. In conclusion, our data indicate that a complex mixture of soluble mediators released by mast cells can enhance tumor growth. This concept is supported by our xenograft experiments showing that mast cells co-injected with thyroid cancer cells can promote tumor proliferation, and the inhibition of mast cell by Cromolyn significantly decreases tumor growth. Thus, anticancer strategies targeting mast cells or their mediators may display therapeutic efficacy in patients harboring malignant thyroid diseases.

Our data also raise several questions. First and foremost, are mast cells an absolute requirement for thyroid carcinoma formation or are they only necessary for tumor progression? We plan to conduct experiments involving mice with genetically defective mast cell development to clarify this issue.

Materials and methods

Tissue samples and immunohistochemistry

Retrospectively collected archival thyroid tissue samples from 96 patients affected by PTCs were retrieved from the files of


a
Tumor volume (mm3)
Weeks
8505-C + HMC-1
8505-C
HMC-1
b
8505-C
8505-C + HMC-1
α-tryptase (40x)
α-CD34 (40x)
α-ki67 (40x)
c
8505-C/GFP
8505-C/GFP + HMC-1
α-ki67
α-GFP
GFP + cells
Percentage of ki67 + cells
p<0.05
8505-C
8505-C + HMC1
d
Tumor volume (mm3)
Weeks
8505-C + HMC-1 + vehicle
8505-C + HMC-1 + Cromolyn
8505-C + vehicle
8505-C + Cromolyn
Cromolyn treatment
e
8505-C + HMC-1 + vehicle
8505-C + HMC-1 + Cromolyn
α-ki67 (40x)
Percentage of ki67 + cells
p=NS
p<0.05
p<0.05
8505-C + vehicle
8505-C + Cromolyn
8505-C + HMC1 + vehicle
8505-C + HMC1 + Cromolyn

the Pathology Department of the University of Pisa on informed consent. Normal thyroid tissue samples were also retrieved from the files of the same department. We used an anti-human tryptase antibody (Dako, Glostrup, Denmark) for mast cell staining. Tryptase expression was counted in 10 high power fields and was scored as follows: 0, absence of positive cells; +, 1–20 cells; ++, 21–50 cells; +++, >50 cells. Anti-ki-67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD34 (Novocastra Lab, Newcastle, UK) antibodies were used to evaluate cell proliferation and vessel density in xenografts. For coimmunostaining of xenografts with anti-ki67 and anti-GFP antibodies, we used the Benchmark semiautomated system (Ventana Medical System, Temecula, AZ, USA). Specimen sections were incubated with a rabbit monoclonal anti-ki67 antibody (Ventana Medical System) and after extensive washing with a mouse monoclonal anti-GFP antibody (Cell Signaling, Danvers, MA, USA). After incubation with biotinylated anti-IgG antibodies, they were visualized with the ultraView Universal Alkaline Phosphatase Red detection kit (Ventana Medical System).

Cell lines

Human primary cultures of normal thyroid cells were maintained according to Curcio *et al*. (1994). Human thyroid cancer cell lines TPC1, 8505-C and NIM were maintained as previously described (Visconti *et al*., 1997; Salvatore *et al*., 2006). HMC-1 and LAD2 mast cell lines were generous gifts from JH Butterfield and AS Kirshenbaum, respectively, and were grown as previously described (Butterfield *et al*., 1988; Kirshenbaum *et al*., 2003). Jurkat cells were obtained from ATCC (Manassas, VA, USA) and cultured as described (Schneider *et al*., 1977). Stable transfection of 8505-C cells with a GFP-expressing vector was performed by electroporation. After G418 selection, GFP-expressing cells were obtained by fluorescence-activated cell sorting analysis.

Experiments in nude mice

To verify that mast cells enhanced tumor growth, we used a xenograft model. Briefly, three groups of 10 mice (4-week-old male BALB/c nu/nu mice; Jackson Laboratories, Bar Harbor, ME, USA) were inoculated subcutaneously into the right dorsal portion with 8505-C cells (10×10^6 per mouse), HMC-1 (1×10^6 per mouse) or a mixture of both. When indicated, xenografts of 8505-C or 8505-C + HMC-1 were treated with sodium cromoglycate (Cromolyn, Sigma, St Louis, MO, USA) or left untreated. Cromolyn (10 mg/kg) dissolved in saline was administered by intraperitoneal injection daily, starting when tumors reached the volume of 40 mm^3 and continuing for the duration of the experiment. Tumor diameters were measured at regular intervals with a caliper. Tumor volumes (V) were calculated with the formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). To evaluate whether circulating mast cells were recruited into tumors, we injected 20 mice with 8505-C cells. When tumors had reached the volume of 40 mm^3, 10 mice were intravenously injected with HMC-1 cells (1×10^6 per mouse). Mice were then killed at days 1, 2 and 7 after injection, and tumor specimens were analyzed for mast cell recruitment and cell proliferation by immunohistochemistry. This study was conducted in accordance with Italian regulations for experimentation on animals.

Isolation and purification of human lung mast cells

Macroscopically normal lung parenchyma was dissected and minced into a single-cell suspension (de Paulis *et al*., 2006). Lung mast cells were purified by countercurrent elutriation (J2/21; Beckman, Brea, CA, USA) and by discontinuous Percoll density gradient and further purified by using an anti-CD117 conjugated to magnetic beads (MACS System; Miltenyi Biotec, Bergisch Gladbach, Germany). The final preparations contained >95% viable cells, assessed by the Trypan blue exclusion method, and purity was >98% mast cells. Purity was assessed by light microscopic observation of stained cells with Toluidine blue, and surface-stained IgE fluorescence antibody.

Statistical analysis

To compare xenograft growth in nude mice, we used the unpaired Student's t-test (normal distributions and equal variances). The clinicopathological data and their correlation with mast cell infiltrate density were statistically evaluated with a two-tailed Student's t-test; the χ^2-test was used to establish the statistical significance of the distributions. Statistical analysis was carried out with the GraphPad Instat software (La Jolla, CA, USA), version 3.0b. Differences were statistically significant at $P < 0.05$.

Cell motility

In vitro cell chemotaxis and invasiveness through Matrigel was assayed using transwell cell culture chambers as described elsewhere (Castellone *et al*., 2004).

RNA extraction and reverse-transcription PCR

Total RNA was isolated by the RNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Detailed experimental procedures can be found in Supplementary information.

ELISA assay

Cytokine levels were measured by quantitative immunoassay ELISA kit (QuantiKine Colorimetric Sandwich Assay,

Figure 8 Enhancement of 8505-C xenografts growth in nude mice in the presence of mast cells (**a**) Xenograft growth of 8505-C thyroid cancer cells in the presence and in the absence of the HMC-1 mast cell line was monitored by measuring tumor size weekly. The addition of HMC-1 to 8505-C cells caused a marked increase in tumor volume. HMC-1 alone cells did not form tumors. (**b**) The expression of tryptase, ki67 and CD34 in xenografts was evaluated by immunohistochemistry. Tryptase positivity indicated that mast cells survived in mixed xenografts. Ki67- and CD34-positive cells were more abundant in mixed than in 8505-C xenografts. (**c**) Co-staining of 8505-C/GFP xenografts with anti-ki67 and anti-GFP shows enhanced proliferation of thyroid cancer cells (GFP^+) in the presence of mast cells. GFP and ki67 staining is shown, respectively, in red and brown. The percentage of GFP^+ proliferating cells in 8505-C/GFP and in mixed xenografts is also shown. (**d**) Xenograft growth of 8505-C and 8505-C + HMC-1 cells in animals treated with vehicle or Cromolyn. The treatment with Cromolyn caused a marked decrease in 8505-C + HMC-1, but not in 8505-C, tumor volume after 2 weeks of treatment. (**e**) Ki67 staining of vehicle- or Cromolyn-treated 8505-C + HMC-1 xenografts. In the bar graph, the rate of proliferation in xenografts is expressed as the percentage of $ki67^+$ cells. Cromolyn treatment did not affect 8505-C xenograft growth, but significantly inhibited the proliferation of 8505-C + HMC-1 mixed xenografts.

ELISA; R&D Systems Europe, Abingdon, UK). Detailed experimental procedures can be found in Supplementary information.

S-phase entry
S-phase entry was evaluated by BrdU incorporation and indirect immunofluorescence. Detailed experimental procedures can be found in Supplementary informations.

TUNEL assay
For apoptosis evaluation, a terminal desoxynucleotidyl transferase-mediated desoxyuridine triphosphate nick-end labeling (TUNEL) was used. Detailed experimental procedures can be found in Supplementary information.

Purification of peripheral blood lymphocytes
Isolation of PBL was performed with standard procedures. Detailed experimental procedures can be found in Supplementary information.

Histamine release
Histamine release was performed by a histamine ELISA kit (IBL, Hamburg, Germany). Detailed experimental procedures can be found in Supplementary information.

Conflict of interest

The authors declare no conflict of interest.

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