High expression of IL-13 receptor α2 in colorectal cancer is associated with invasion, liver metastasis and poor prognosis

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ABSTRACT

Autocrine secretion of cytokines by metastatic colorectal cancer cells and their role during invasion and liver homing has been poorly characterized. In this study, we used cytokine arrays to analyze the secretomes of poorly and highly metastatic colorectal cancer cells. Compared with poorly metastatic cancer cells, highly metastatic cells expressed increased levels of the immunosuppressive cytokines IL-4 and IL-13 in addition to increased surface expression of the high affinity IL-13 receptor IL13Rα2, suggesting that IL13Rα2 mediates IL13 effects in colorectal cancer cells. Silencing of IL13Rα2 in highly metastatic cells led to a decrease in adhesion capacity in vitro and a reduction in liver homing and increased survival in vivo, revealing a role for this receptor in cell adhesion, migration, invasion and metastatic colonization. In support of this, IL-13 signaling activated the oncogenic signaling molecules PI3K, AKT, and SRC in highly metastatic cells. Clinically, high expression of IL13Rα2 was associated with later stages of disease progression and poor outcome in colorectal cancer patients. Our findings therefore support a critical role for IL13Rα2 expression in colon cancer invasion and metastasis.

Precis:

Signaling through a cytokine decoy receptor is found to play a critical role in the adhesion, invasion and colonization of highly metastatic colorectal cancer cells, with potential implications for their therapeutic management.

Abbreviations: IL13Rα2, interleukin 13 receptor alpha 2; RT-PCR, reverse transcriptase polymerase chain reaction; shRNA, short hairpin RNA; TMA, tissue microarray
Introduction

Metastasis is the final step of the malignant transformation, being responsible for the fatal outcome in cancer patients. However, metastasis is a complex process that involves a number of different and sequential steps. Cells must detach from the primary tumor, migrate, gain access to blood or lymphatic vessel and colonize a new target organ (1). Therefore, metastasis cannot be considered as a single process, but as a collection of different events, all of them exhibiting different molecular traits. Migration, invasion and survival are fundamental aspects of the process.

Dissemination and metastasis of cancer cells depend on extensive interactions within the tumor microenvironment (2-3). A number of these interactions are regulated through chemokines and their receptors, which govern many different aspects of the malignant phenotype. So, it is critical to characterize chemokines and their receptors expression in metastatic cells. Chemokine-mediated inflammation has been shown to play an important role in tumor biology by influencing tumor growth, invasion and metastasis (4). Chemokines were initially described as regulators of leukocyte trafficking and recruitment in inflammation sites. Infiltration of white blood cells, mostly tumor-associated macrophages, and the presence of pro-inflammatory molecules as IL-8 and IL-6 are typical features of cancer-related inflammation. Cytokines and chemokines are produced by multiple cell types, including fibroblasts, endothelial and epithelial cells (5), including cancer cells, which also express their G-protein coupled receptors. These G-proteins activate a number of signaling pathways such as PLC-β, PI3K and the MAPK cascade, which play a critical role in adhesion, migration and survival of cancer cells (6).
Concomitant with these effects, cytokines and chemokines also confer cancer cells with the ability to adhere to fibronectin and collagen IV, allowing the formation of micrometastases (7). Therefore, chemokine networks control critical steps of the adhesion-invasion-metastasis cascade, providing the necessary signals that facilitate cancer cell survival and growth in distant sites (3). Little is known about the role played by the autocrine secretion of chemokines by tumor epithelial cells in cancer-related inflammation, which might regulate the recruitment and maturation of macrophages and other effector cells. We used the well-known KM12 cell model for the study of late metastatic events in colorectal cancer, including liver colonization and survival of the tumor cells in the new environment. KM12C and KM12SM epithelial cells differ only in their metastatic properties (8). KM12SM, a highly metastatic cell line, was derived from the poorly metastatic cell line KM12C, through successive passages in nude mice. A previous proteomic characterization of these two colon cancer cell lines showed a preference for expression of homing molecules in KM12SM cells (9). Moreover, a decrease in the expression levels of enzymes from the glycolysis, pentose phosphate pathway and amino acid transporters was observed.

To characterize the metastatic process in colorectal cancer, we analyzed the cytokine/chemokine profiles released by colorectal metastatic cancer KM12SM cells using antibody microarrays. These microarrays constitute a powerful tool to get a complete overview of cytokine profiles and pathways alterations in cancer (10). We observed higher secretion of IL-13, which was associated to a more abundant expression of their receptor IL13Ra2 in the highly metastatic KM12 SM cells. Silencing of IL13Ra2 revealed a major role for this receptor in cell adhesion, migration, invasion and metastatic colonization of colorectal cancer cells. A clear increase of expression of IL13Ra2 was observed in late stage human colon cancer tissues, showing
a correlation between IL13Ra2 expression and poor outcome of patients with colon cancer metastasis.
MATERIALS AND METHODS

Cell culture

KM12C and KM12SM human colon cancer cells were obtained directly from I. Fidler’s lab (MD Anderson Cancer Center, USA). KM12 cells were expanded in the laboratory to prepare a large batch of working aliquots that were stored in liquid nitrogen. For each experiment, cells were thawed and kept in culture for a maximum of 10 passages. These two cell lines were not authenticated in our laboratory. Parental cells, and their derivatives, were cultured in DMEM (Gibco-Life Technologies) containing 10% fetal calf serum and antibiotics at 37°C in a 5% CO2-humidified atmosphere. Other cell lines were purchased directly from the American Type Culture Collection (ATCC) and cultured as recommended. All these cell lines were authenticated by ATCC and were passaged fewer than 6 months after purchase for all the experiments.

Human Protein Cytokine Array.

Conditioned medium from each KM12 cell type was collected after 24 and 48h in serum-free medium and incubated with arrays containing 79 human cytokine specific antibodies as described (10). Then, the membranes were scanned and analyzed using Redfin, a 2D-gel image analysis software (Ludesi). Relative cytokine intensities were normalized in comparison to control spots on the same membrane. Ratios between both cell lines were calculated for the different cytokines at the three experiments. Individual quantification of IL-4 and IL-13 was carried out using specific ELISA kits (RayBiotech) (see Supplementary Methods).

Stable transformation of KM12 cells, cell adhesion and invasion assays

pLKO.1 vectors containing 5 different shRNAs for IL13Rα2 were purchased from Open Biosystems. As shRNA control vectors, we used a scrambled shRNA and an empty pLKO.1 vector obtained from AddGene (11-12). Stable transformed cells were obtained by lentiviral infection. Briefly, HEK293T cells were transfected with pLKO.1 vectors and the packaging
vectors pCMV-rev, pMDLg-pRRE and pNGVL-VSVG using jetPRIME Transfection Reagent (Polyplus). After incubating the cells for 12-15 h in serum-free medium, medium was replaced with DMEM containing 10% FBS and antibiotics. The day after, conditioned medium containing lentiviral particles was centrifuged, diluted 1:2-1:10 in DMEM containing 10% FBS and antibiotics and directly added to KM12C and KM12SM cells. After three days of incubation, infected KM12C and KM12SM CRC cells were selected using 1 \( \mu \text{g/ml} \) puromycin (Sigma) during 2-3 weeks. Then, cells were cultured with medium containing 0.5 \( \mu \text{g/ml} \) puromycin.

Cell adhesion and invasion using Matrigel assays were carried out according to previously published procedures (13) (see Supplementary Methods for a full description). Inhibitors LY294002 and PP2 were from Sigma and UO126 was from Calbiochem. For apoptosis and proliferation assays see Supplementary Methods.

**Western blot**

The following antibodies were used in the study. Anti-IL4R\( \alpha \), anti-IL13R\( \alpha \)1 and anti-\( \alpha \)2 integrin were from Abcam, whereas anti-IL13R\( \alpha \)2 was from R&D Systems. AKT, pAKT, pERK1/2, pJNK, STAT3, pSTAT3, STAT6 and pSTAT6 were from Cell Signalling. ERK1/2, JNK and RhoGDI were from Santa Cruz Biotechnology. Tubulin was from Sigma. Anti-\( \beta \)1 integrin was a gift from Dr F. Sánchez-Madrid (H. de la Princesa. Madrid. Spain).

For western blot, KM12C and KM12SM cells were washed twice with chilled PBS and homogenized in 0.05% SDS. Protein extracts were sonicated three times for 30s on ice, and clarified at 10000g at 4 \( ^\circ \)C. Protein extracts were run in 10% SDS-PAGE and immunoblotting was performed following standard procedures.

**Metastasis experiments in nude mice.**

Swiss nude mice (Charles River) were used for intra-splenic or intra-tail inoculation. The Ethical Committee of the Consejo Superior de Investigaciones Científicas (Madrid,
Spain) approved the protocols used for experimental work with mice. Mice were injected with $10^6$ KM12 cells in 0.1 ml PBS. Mice were daily inspected for signs of disease, such as abdominal distension, locomotive deficit, or tumor detectable by palpation. Metastasis nodes in liver were determined by counting the number of visible nodules in dissected livers.

**Immunohistochemistry analysis**

A total of 80 patients diagnosed and treated of colorectal adenocarcinoma between 2001 and 2006 in Fundación Jiménez Díaz (Madrid) were used for the study. We have reviewed the clinical records of the patients to determine tumor stage at the time of diagnosis and outcome (Supplementary Table S1). Hematoxylin-eosin stained sections of the colectomy specimens were reviewed to select representative areas of the tumor to perform immunohistochemical detection of IL13 and IL13Ra2. The working dilution was 1/100 for IL13 and 1/500 for IL13Ra2. IL13Ra2 immunohistochemistry was performed on a tissue microarray (TMA) using an automated system for immunostaining (Dako Autostainer), with antigen retrieval at high pH. The immunostained sections were counterstained with hematoxylin and the intensity of the membrane and cytoplasmic staining was graded as absent, weak, moderate or intense, although for subsequent statistical analysis the cases were reclassified as positive (either moderate or intense) or negative (either complete absence of positivity or weak staining similar to control areas of normal colonic mucosa). In all cases, sections from normal colonic mucosa distant from the tumor site were used as negative controls.

**Statistical analyses.**

Data from the patients were recorded in an Excel file. Descriptive statistics included mean (and standard deviation) for quantitative Gaussian variables and percentages for qualitative ones. Bivariant analysis was made with one-way ANOVA followed by
Tukey-Kramer multiple comparison test for quantitative variables and chi-square test for qualitative ones. In both analyses the minimum acceptable level of significance was \( p < 0.05 \). Survival curves were plotted with Kaplan-Meier technique and compared with the log-rank test. The level of significance for survival was also settled at a \( p \) value \( < 0.05 \).
RESULTS

Chemokine profiling in the secretome of KM12 cells

We investigated the production and release of 79 different chemokines, growth factors and other immunomodulators in conditioned culture media of colorectal cancer KM12 cells by using a chemokine array. Results after 24 and 48h of culture are shown in Fig. 1A. Several chemokines, pro-angiogenic and growth factors were highly expressed in both cell lines, among them GRO\textsubscript{α} (CXCL1), IL-8 (CXCL8), Rantes (CCL5), MIP-1β (CCL4), Eotaxin-2 (CCL24), angiogenin, MIF, osteoprotegerin or IGFBP2 (Fig. 1B).

Regarding differential expression, TIMP2, IL-4 and IL-13 were up-regulated in the highly metastatic cells (Fig. 1A, B). IL-4 and IL-13 differences were confirmed by using a specific ELISA, with fold-changes between 1.5-2.13, in the presence or absence of serum, respectively (Fig. 1C).

Most of the biological effects of IL-4 and IL-13 are mediated by STAT transcription factors (14). We analyzed KM12 cells by western blot using antibodies specific for total and phosphorylated forms of STAT3 and STAT6 (Fig. 1D). Total and activated STAT6 were more abundant in KM12SM cells, with no significant differences in STAT3. High levels of STAT6 in colon cancer cells have been associated to high invasiveness/metastasis (15). These results support a potential action of IL-4 and IL-13 on KM12 cells.

IL13R\textsubscript{α}2 mediates IL-13 action on KM12 cells. Effects on cell adhesion

IL-4 and IL-13 usually regulate biological activity through the common type II receptor IL4R\textsubscript{α}/IL13R\textsubscript{α}1 complex (16), which mediates signal transduction through the JAK-STAT6 pathway (17). In addition, IL-13 binds a high-affinity receptor called IL13R\textsubscript{α}2
(18), which was initially assigned as a decoy receptor (19). We tested KM12 cells for
the presence of these receptors. IL4Rα was not detected on the surface of KM12 cells
by flow cytometry (Supplementary Fig. S1A) nor intracellular by western blot
(Supplementary Fig. S1D). IL13Rα1 was not accessible on the cell surface
(Supplementary Fig. S1B), although some intracellular expression was detected using
permeabilized cells in flow cytometry (Supplementary Fig. S1C) or western blot
(Supplementary Fig. S1E). In contrast, a higher surface expression of IL13Rα2 was
observed on the surface of KM12SM than in KM12C cells by flow cytometry, 73.9%
vs. 33.9% (Fig. 2 A). Western blot analyses confirmed the increased expression of
IL13Rα2 in KM12SM cells respect to KM12C, as well as a high expression in other
metastatic colon cancer cells such as SW48 or HT29 (Fig. 2 B). Since the
IL4Rα/IL13Rα1 heterodimer for IL13 signaling was not available on the cell surface,
we hypothesized that IL13Rα2 could mediate IL-13 action in KM12 cells.

To examine the potential effect of IL13Rα2 on colorectal cancer metastasis,
IL13Rα2 was silenced on KM12C and KM12SM cells by preparing stable shRNA
transfectants. The lack of expression of IL13Rα2 was confirmed by western blot (Fig.
2C) and semi-quantitative RT-PCR (data not shown). Both analyses showed that less
than 10% of the original protein expression levels were detected after knockdown with
shRNA#23. This silencing did not affect the expression of IL13Rα1, which showed
similar intracellular levels (Supplementary Fig. S1E). To analyze the effect of
IL13Rα2-silencing on autocrine IL-13 production we quantified the levels of IL-13 by
ELISA. We observed a decrease in the expression of IL-13 in IL13Rα2-silenced cells.
The reduction was more dramatic for KM12SM cells (Supplementary Fig. S2).

Then, we analyzed the adhesive properties of the cell lines before and after
IL13Rα2-silencing using Matrigel. In basal conditions, adhesion capacity of scrambled
KM12SM overexpressing IL13Rα2 duplicated that of KM12C cells. The decrease in adhesion capacity after IL13Rα2 silencing was particularly significant for KM12SM cells (>50%) and negligible for KM12C cells (Fig. 2D). Addition of IL-13 increased significantly cell adhesion in scrambled control cells, but not in the silenced cells. The use of anti-IL13Rα2 antibody blocked cell adhesion for IL13-treated scrambled cells, being most relevant for KM12SM (Fig. 2D). Still, antibody blocking was not as effective as IL13Rα2-silencing. These results suggest that IL13Rα2 also contributes to constitutive cell adhesion in the absence of IL-13. As a control, IL13Rα1-silenced cells (Supplementary Fig. S3A) did not show alterations in cell adhesion (Supplementary Fig. S3B).

**IL13Rα2 expression promotes cell migration and invasion in KM12 cells through PI3K and SRC activation**

To determine the effect on cell migration, we used wound healing assays and different amounts of IL-13 for 22h. IL-13 was sufficient to promote migration in KM12 cells, with an optimum at 10 ng/ml (Fig. 3A). KM12SM cells displayed twice more migratory capacity than KM12C cells in presence of serum, which might contain IL-13 and other pro-migratory factors. KM12 cells showed a much lower cell migration in wound healing after IL13Rα2 silencing (Fig. 3B). Addition of IL-13 caused a significant increase in the migration speed of scrambled cells (Fig. 3C). In contrast, silenced cells were insensitive to IL-13. This inhibition was also confirmed by the use of anti-IL13Rα2 antibodies. To study the pathways involved in the increase of cell migration we tested different inhibitors. The increase in migration induced by IL-13 was strongly reduced by LY294002, a PI3K inhibitor, partially reduced by PP2 (SRC inhibitor), and not affected by UO126 (MEK1/2 inhibitor) in KM12 cells. The inhibition was more pronounced on KM12SM cells.
Then, we tested the ability of the cells for invasion across Matrigel (Fig. 3D). In medium alone, the invasion was low, but twice higher for KM12SM cells. Addition of IL-13 caused a large increase of invasion in the control cells, but not in the silenced cells, which showed between six and ten times less invasion capacity, similar to basal levels. As before, the use of PI3K inhibitor or anti-IL13Rα2 decreased the invasive capacity of the scrambled cells to basal level. SRC inhibitor was also quite effective to decrease the invasive capacity of KM12SM, but not so much on KM12C cells. No effect was observed for MEK1/2 inhibitor. As a control, IL13Rα1 silencing in KM12SM cells did not alter cell invasion capacity (Supplementary Fig. S3C).

To confirm the role of IL13Rα2 in cell migration and invasion, we generated KM12 cells overexpressing this receptor, which were tested in migration and invasion assays. Both KM12C and SM transfectants showed an increase in migration and invasion in response to IL-13 compared to control transfectants (Supplementary Fig. S4A-B). Collectively, these results support that IL13 action was mediated by IL13Rα2 for migration and invasion in colorectal cancer metastasis.

**Pathways activation in IL13Rα2-mediated cell invasion**

To study the mechanism of action of IL13Rα2 in cell invasion, we characterized the levels of activated SRC, FAK, AKT, ERK, JNK and STAT6 in response to IL-13 or serum (Fig. 3E). Expression of phospho-SRC and phospho-AKT was more abundant in KM12SM cells treated with serum than with IL-13, whereas only phospho-AKT, but no phospho-SRC, was observed in KM12C cells. These differences in phospho-SRC effect between KM12SM and KM12C cells might explain previous differences in migration and invasion induced by the SRC inhibitor. The presence of a double band with different intensities in phospho-SRC between serum and IL13-treated cells suggests that
a different member from the SRC family might be implicated in KM12SM cells, as the antibody recognizes phosphorylation in the C-terminal tyrosine of six members of this family. The decrease of phospho-AKT in silenced KM12SM cells treated with IL-13 confirmed the relevance of PI3K and suggests a potential effect on survival. MAPK signaling was not affected by IL13Rα2 silencing as no differences were observed for ERK1/2 or JNK. Activation of phospho-STAT6 was observed in KM12SM cells with serum, but not when cells were treated only with IL13 (Fig. 3E). Therefore, IL13-triggered activation through IL13Rα2 is STAT6-independent. Other factors must activate STAT6 in serum-cultured KM12SM cells.

Silencing of IL13Rα2 expression in KM12 cells decreases survival, tumorigenesis and proliferation.

The activation of the PI3K pathway has important biological effects on cell survival and proliferation (20). To test whether IL-13 signaling via IL13α2 can modulate cell survival, KM12 cells were subjected to apoptosis assays. In response to oxidative stress induced by hydrogen peroxide, scrambled KM12C and KM12SM cells showed similar levels of apoptosis (Fig. 4A). The addition of IL-13 caused a moderate effect in promoting survival in these oxidative stress conditions. This effect was dependent on IL13Rα2, as the addition of the antibody or the silencing of the receptor abolished the increment in survival (Fig. 4A). The presence of LY294002, a PI3K inhibitor, inhibited this increase in survival induced by IL-13 through IL13Rα2. These results indicate that IL13 also affects the survival program in KM12 colorectal cancer cells through PI3K-AKT activation mediated by the IL13Rα2 receptor. This moderate reduction in cell apoptosis may play a role during metastasis, facilitating survival of metastatic cells.
Then, we tested the effect of IL13Rα2 silencing on tumorigenesis through the ability to form colonies in soft agar. KM12SM displayed a higher capacity to proliferate in an anchorage-independent environment, when compared to KM12C (Fig. 4B). However, IL13Rα2-silenced cell lines showed a significant reduction in colony formation, indicating a crucial role of this receptor in tumorigenesis. Regarding proliferation, we observed only a minor effect in cells treated with IL-13 (Fig. 4C). This IL-13 effect was blocked after incubation with anti-IL13Rα2 antibodies (Fig. 4C). These results suggest that IL-13 signaling via IL13Rα2 does not play a major role in cell proliferation in colorectal cancer cells.

Silencing of IL13Rα2 in KM12SM cells provokes a decrease in liver homing and an increase in mouse survival

As a final approach to verify the role of IL13Rα2 in colon cancer invasion and metastasis, we carried out intra-splenic injections of control and silenced KM12 cells to analyze their ability and speed to generate metastasis in liver. IL13Rα2-silenced KM12SM cells induced longer survival of mice than control KM12SM cells (p<0.05) (Fig. 5A). This prolonged survival was due to the slower growth of the tumor in the mice inoculated with IL13Rα2-silenced KM12SM cells and lower ability to colonize liver and cause metastasis. Post-mortem analysis showed that tumors at primary inoculation site reached similar sizes ranging between 0.7-1 cm³ in control and silenced KM12SM cells. Poorly-metastatic KM12C cells did not cause metastasis, and only 30% of inoculated mice developed a tumor in spleen 180 days after inoculations. Therefore, IL13Rα2-silenced KM12SM cells lost, in a significant way, the ability to colonize the liver, as few mice showed macroscopic metastasis after liver dissection (Fig. 5B).
To corroborate the ability of KM12 cells for liver homing, we collected the livers 24 h after spleen injection and carried out a PCR for amplification of human GAPDH (Fig. 5C). Whereas human GAPDH could be detected in livers from mice inoculated with control cells (especially KM12SM), the PCR amplification resulted in a barely detectable band in mice inoculated with silenced cells. To examine IL13Rα2 expression in KM12SM cells as well as their invasive properties after in vivo passage, cells were isolated from the tumors and cultured until confluence. IL13Rα2 expression remained very low (Fig. 5D) and their invasiveness across Matrigel was not altered by in vivo passage (Fig. 5E). Collectively, these data confirm the capacity of IL13Rα2 to mediate homing and liver metastasis in colon cancer.

**IL13Rα2 overexpression in human patients is associated to late stages and lower overall survival**

To investigate the relevance of our results in human colon cancer, we decided to study the levels of expression of IL-13 and IL13Rα2 in tumor and adjacent normal tissue samples. We observed IL-13 expression mainly associated to human epithelial colon cancer cells, with weak or no IL-13 expression in the stroma of the tumors (Fig. 6A). For statistical analysis of IL13Rα2 expression, we used a tissue microarray (TMA) with representative sections of tumor and normal colonic mucosa from 80 patients diagnosed and treated of colorectal adenocarcinoma and followed on the long term (more than five years). The series was retrospectively selected. IL13Rα2 expression was not detected in 27 cases (33.7 %) and was present with either moderate or high intensity in the remaining 53 cases (66.3%) (Fig. 6 B). Weak or no-staining was observed in all control normal samples. We found a statistically-significant association between IL13Rα2 expression and tumor progression (T stage), with higher expression in T3 or T4 tumors as compared with T1 or T2 (p = 0.013), lymph node involvement (higher expression in
tumors with lymph node involvement, \( p = 0.013 \) and metastasis at the moment of
diagnosis (\( p = 0.038 \)) (Fig. 6C). We found no significant association with histological
grade of the tumor, but all high grade and colloid tumors showed intense IL13R\( \alpha_2 \)
expression, as opposed to well-differentiated tumors, that were mostly negative.
Finally, we wanted to know if there was an association between IL13R\( \alpha_2 \) expression
and survival of colon cancer patients. Survival analysis showed a clear association with
poor prognosis in terms of lower overall survival for patients with high IL13R\( \alpha_2 \)
expression (\( p = 0.03 \)) (Fig. 6D).
Discussion

Although the KM12 cell model probably does not give a complete picture of the spontaneous metastasis in human colon cancer, actually is giving us excellent insights in the cell adhesion, invasion and colonization of the liver in metastasis and the critical molecules involved in these processes. Here, we have described an important role for IL-13 and its receptor IL13Rα2 in colorectal cancer invasion and metastasis. This conclusion was obtained from the following observations: i) IL-13 was more abundant in the secretome of highly metastatic cells, ii) IL13Rα2 was overexpressed in highly metastatic KM12SM cells and other metastatic cell lines compared to poorly metastatic KM12C cells, iii) IL13Rα2-silencing decreased adhesion, invasion and clonogenicity, iv) IL13Rα2-silencing suppressed AKT activation and promoted apoptosis, v) mice experiments demonstrated that removal of IL13Rα2 reduced the homing capacity in liver of KM12SM cells and increased the survival of inoculated mice and vi) human colon cancer samples showed a high expression of IL-13 and IL13Rα2 in cancer cells. IL13Rα2 was mainly associated to T3 or T4 tumors and to a lower overall survival. These results confirmed that IL-13 and IL13Rα2 expression were associated to colorectal cancer invasion and liver metastasis in cancer cells.

IL-13 has been previously associated to pathological conditions such as asthma, autoimmune diseases and inflammatory conditions (21). It has been involved in Th2 differentiation, STAT6-dependent M2 polarization and TGF-β1 production and fibrosis (22-23). Stimulation of mouse J774 cells with IL4 or IL-13 in combination with TNF-α induced STAT6 phosphorylation (14) and abundant expression of IL13Rα2 on the surface of the cells (23). IL-13 acts as an autocrine growth factor in pancreatic cancer that promotes lymph node metastases (24) and is a major regulator of M2 macrophages to suppress immune surveillance in metastasis (25). This counter-surveillance activity
requires the expression of IL13Rα2 (26). IL13Rα2 gene expression was reported in pancreatic and breast cancer metastasis (27-28). The almost absence of IL4Rα and IL13Rα1 in KM12 cells (this report) and other colon cancer cells (29) indicates that IL-13 signaling occurs through the IL13Rα2 receptor. Surprisingly, previous reports did not detect IL13Rα2 mRNA by RT-PCR in HT-29/B6 colon cancer cells (30).

No signaling activity was initially described for IL13Rα2 due to its short cytoplasmic domain and the lack of JAK/STAT binding sequences (19). Therefore, IL13Rα2 was considered as a decoy receptor for IL-13 in mouse and humans (21). However, recent studies have demonstrated that IL13Rα2 is internalized after IL-13 binding (31). It has been reported that IL13Rα2 induces MAPK signal transduction in intestinal epithelial cells from ulcerative colitis or colorectal cancer (29) and pancreatic cancer (28). At low concentrations, IL13Rα2 signaled through the MAPK pathway, but at higher concentrations worked as a decoy receptor (29). In contrast, on metastatic KM12SM cells, IL-13 action is mediated through IL13Rα2 independent from the expression levels, as denoted by the effect on both, KM12C and KM12SM cells.

In murine macrophages, IL13Rα2 mediates AP-1 dependent, STAT-6 independent signaling, resulting in inflammation and fibrosis in vivo (23). Previous reports in pancreatic cancer (28) and ulcerative colitis (29) showed STAT6-independent activation in IL13Rα2 expressing cells, suggesting an inverse relationship between IL13Rα2 expression and STAT6 activation. In colon cancer metastatic cells, signaling through IL13Rα2 is also STAT6-independent, and is not mediated by JNK or ERK. The lack of ERK activation could explain the minor effect of IL13Rα2 removal on cell proliferation and might explain why IL13Rα2 overexpression does not provide any competitive advantage in subcutaneously implanted tumors in immunodeficient mice (32).
In our model, IL13Rα2 signaling induced PI3K activation, as PI3K inhibitors blocked IL-13 effects on KM12 cells. SRC activation was found exclusively on IL13-treated KM12SM cells and might be related with the higher migration and invasion of these cells. An alternative role for IL-13 in the increase of intestinal epithelial permeability, mediated by the PI3K pathway without STAT6 involvement, has been reported (33). Ulcerative colitis, a well-known risk factor for colon cancer development, is probably driven by IL-13 (34). Also, oxazolone-induced colitis in mice is caused by IL13-producing natural killer T cells (35). Lack of epithelial barrier function has been reported for inflammatory bowel diseases (36-37). This increase in permeability affected epithelial cells tight junctions, induced epithelial apoptosis and cell restitution arrest in ulcerative colitis (30). All these processes, where IL-13 is a key effector, contribute to severe inflammation and could constitute a connecting link between ulcerative colitis, colon inflammation and colon cancer. In fact, we have observed a predominant association of IL-13 expression with colon cancer epithelial cells.

Immunohistochemical analysis of IL13Rα2 expression in a panel of metastatic human colon cancer samples showed a clear association with late stages in human cancer and poor outcome of patients. This worst prognosis could be attributed to the increased invasiveness and homing capacity of cells overexpressing IL13Rα2. Liver colonization by metastatic cells requires a different program of protein expression and signaling activation from early metastasis. Targeting some of the cytokines or regulatory molecules involved in metastatic invasion and homing might be a successful approach for decreasing metastasis recurrence in colorectal cancer.

In summary, we have demonstrated a role for IL13Rα2 in adhesion, invasion, survival and colonization of highly metastatic colon cancer cells. Our results suggest
that IL13Rα2 does not function only as a decoy receptor of IL-13 in cancer cells but as signaling mediator. Moreover, the use of IL13Rα2 expression in colon cancer patients as a prognostic biomarker in metastatic colorectal cancer gives a pathophysiological relevance to these findings and supports the interest of this molecule as therapeutic target in colon cancer.
REFERENCES


LEGENDS TO FIGURES

Figure 1. Expression of chemokines, growth factors and immunomodulators in conditioned medium from KM12SM and KM12C cells using cytokine arrays. A) Representative image of cytokine antibody arrays results after screening of conditioned medium from KM12 cells. The experiment was carried out in triplicate. B) Cytokines highly expressed in KM12 cells at 48 h. Bar graph was calculated for each cytokine with the median value in arbitrary units of three independent assays. Inset: Signal intensity for IL-4 and IL-13 obtained from three independent arrays. C) Expression ratios for IL-4 and IL-13 in conditioned medium of KM12 cells in the presence or absence of serum at 48 h after ELISA quantification. D) STAT-signaling alterations in KM12 cells were tested by immunoblotting. Experiments were performed in triplicate and quantified by densitometry.

Figure 2. IL13Rα2 is overexpressed on KM12SM cells and increases cell adhesion. A) Flow cytometry analysis of KM12C and KM12SM cells. The percentage of positive cell is shown inside each panel. B) 25 µg of protein from lysates of the indicated colorectal carcinoma cell lines were resolved in polyacrylamide gels and subjected to Western blot using anti-IL13Rα2 or, as a control, anti-RhoGDI antibodies C) KM12C and KM12SM cells were infected with retroviral vectors containing different shRNAs targeting IL13Rα2, scrambled shRNA or empty vectors, and IL13Rα2-expression was assessed by western blot. Bands were quantified with MultiGauge software. Tubulin was used as loading control. D) Cell adhesion to Matrigel of IL13Rα2-silenced or control KM12 cells, pretreated for 5h with or without IL-13 plus the indicated antibodies. Adhesion was significantly upregulated by incubation with IL-13 (** p<0.01, *** p<0.001) and significantly decreased by incubation with anti-IL13Rα2 (ΔΔ p<0.01, ΔΔΔ p<0.001). Data represent the mean ± SD of 3 independent experiments.
**Figure 3. IL-13 increases cell migration and invasion through IL13Rα2.** A) Cultures were incubated in presence of the indicated concentrations of IL-13. Migration speed of the cells was calculated as the distance covered in 48h. B) Wound healing assay of KM12C and KM12SM cells incubated in medium alone or with 10% serum. Pictures were taken 48 h after scratching. C) KM12 cells were incubated in presence or absence of IL-13 (10 ng/ml) with the indicated antibodies and inhibitors. D) Invasion across Matrigel of the KM12 cell lines treated as indicated. Data represent the mean ± SD of 3 independent experiments. For increase in migration (* p<0.05, ** p<0.01, *** p<0.001) and for reduction (Δ p<0.05, ΔΔ p<0.01, ΔΔΔ p<0.001). E) KM12 cells were starved in medium, treated with IL13 (10 ng/ml) or 10% serum for 5 h and lysed. The extracts were analyzed by western blot using the indicated antibodies. * indicates observed changes in expression.

**Figure 4. IL13Rα2 promotes cell survival and proliferation.** (A) Cells were incubated with H₂O₂ for 16 h in presence of medium alone or with IL-13, and in presence or absence of LY294002 inhibitor or anti-IL13Rα2 antibodies, and subjected to apoptosis detection assays. (B) Colony formation assay in soft agar with the indicated cell lines. C) Proliferation and cell viability was determined by MTT assays after 24-48 h of incubation in medium with or without IL-13 (10 ng/ml) and anti-IL13Rα2 antibody. Optical density was significantly increased by addition of IL-13 (* p<0.05, ** p<0.01) and significantly inhibited by anti-IL13Rα2 (Δ p<0.05, ΔΔ p<0.01).

**Figure 5. IL13Rα2 promotes liver metastasis of KM12SM cells.** A) Kaplan-Meier survival assay of nude mice inoculated with the indicated KM12 cells. Survival of mice inoculated with IL13Rα2-silenced KM12SM cells increased significantly (*, p<0.05) when compared to those inoculated with scrambled cells. B) Mice were examined for macroscopic metastases in liver. Number of macroscopic metastases in liver was
significantly reduced in mice inoculated with IL13Rα2-silenced KM12SM cells (**, p<0.01). ◇ (Diamonds): number of metastases in each mouse. Average and standard deviation are shown. C) Nude mice inoculated as in A were sacrificed 24 h after inoculation. RNA was isolated from the liver and subjected to RT-PCR to amplify human GAPDH. A representative experiment out of three is shown. Murine β-actin was amplified as loading control. D) KM12 cells isolated from tumors by mechanical disaggregation were cultured to confluence and analyzed by western blot to confirm IL13Rα2-silencing and E) tested for invasion through Matrigel in presence or absence of IL-13 (10ng/ml). Silenced KM12SM cells were included in the assay as a control.

**Figure 6. IL13Rα2 expression is associated to human colon cancer progression and poor survival of colorectal cancer patients.** A) Immunohistochemical analysis of IL-13 expression in human CRC tissue showed that IL-13 expression occurs preferentially in CRC epithelial cells. B) Immunohistochemical analysis of IL13Rα2 expression in tissue microarrays showing representative images of strong, moderate or negative staining of different colon carcinomas and negative normal paired mucosa. Counterstaining was made with hematoxylin. Pictures were taken at x200 magnification. C) Quantification of IL13Rα2 expression by estimation of staining intensity was performed as described in Methods. Eighty samples were analyzed. Positive and negative values were represented as bar graphs. p values were calculated with the chi-square test. D) Kaplan-Meier analyses of overall survival of cancer patients according to the expression of IL13Rα2. Significant association of IL13Rα2 expression with lower overall survival was found by comparing differences between curves with the log-rank test.
Figure 1

A) Western Blot (WB) analysis of STAT6 and pSTAT6 expression in KM12C and KM12SM cells after 24h and 48h treatment with or without serum. 

B) Cytokine profile comparison between KM12C 48h and KM12SM 48h, showing increased levels of IL-4 and IL-13 in KM12SM compared to KM12C.

C) ELISA assay showing upregulation of IL-4 and IL-13 in KM12SM compared to KM12C.

D) Western Blot (WB) ratio of KM12SM/KM12C for STAT3, pSTAT3, STAT6, pSTAT6, osteoprotegerin (OPG), osteinostatin M, and Tubulin, with increased expression in KM12SM compared to KM12C.
Figure 2

A. KM12C and KM12SM cell number histograms showing mean fluorescence intensity.

B. Western blot analysis of IL13Ra2 and RhoGDI expression in non-metastatic and metastatic cell lines.

C. Gel image showing shRNA silencing of IL13Ra2 in KM12C and KM12SM cells.

D. Bar graph showing adhesive cell count for KM12C and KM12SM cells in medium, IL13, medium + anti-IL13Ra2, and IL13 + anti-IL13Ra2 conditions.
**Figure 3**
Figure 4
Figure 5
Figure 6
High expression of IL-13 receptor α2 in colorectal cancer is associated with invasion, liver metastasis and poor prognosis

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