TIM-3 Is Expressed in Melanoma Cells and Is Upregulated in TGF-Beta Stimulated Mast Cells

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Many studies detect elevated numbers of mast cells in tumors, but it is still controversial whether they are beneficial or detrimental for tumor cells. Furthermore, many tumors, such as melanomas, produce large quantities of transforming growth factor (TGF)-β and during tumorigenesis the apoptotic and growth-inhibitory effects of TGF-βs are lost. Based on these data we investigated the gene expression changes in TGF-βI-treated human mast cells with DNA microarray and detected 45 differentially regulated genes, among them T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3). As the major sources of TIM-3 ligand galectin-9 are not tumor cells, but rather mast cells, this raises the possibility of an autocrine mechanism resulting in local immunosuppression through the elevated TIM-3 expression by TGF-βI. Interestingly, not only melanoma tissue sections contained TIM-3-positive mast cells, but we detected this protein also in melanoma cells. Furthermore, TIM-3 was expressed in both WM35 and HT168-M1 melanoma cell lines at a higher level than in isolated epidermal melanocytes, which can contribute to the lower adhering capacity of tumor cells. In conclusion, the immunoregulatory molecule TIM-3 in TGF-β-stimulated mast cells and melanoma cells may support the survival of this tumor type.

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INTRODUCTION

Though the function of mast cells was mostly known in allergic immune responses and local hypersensitivity, recently their importance has been shown in multiple immunological, inflammatory, developmental, and malignant processes. Human mast cells are classified based on their protease content. Although the tryptase and chymase-containing mast cell (MC(TC)) subtype expresses and stores chymase, tryptase and cathepsin G and is localized in the skin and the submucosa of gut and lung, the other basic human mast cell type contains only tryptase and hence is called tryptase-containing mast cell (MC(T)). This latter subtype can be regarded as the orthologs of murine mucosal mast cells based on their tissue localization. Interestingly, murine and human mast cells differ in many features such as in their differentiation program, cytokine producing capabilities, or protease content. Although murine mast cells serve as a useful model for several processes, data obtained from these studies cannot be directly transferred to the human system.

Transforming growth factor (TGF)-βI acts on the immune system by multiple ways. It inhibits T-cell proliferation, acquisition of Th cell functions, or differentiation of $CD8^+$ T-cells. TGF-βI also influences macrophages, B-cells, dendritic cells, and contributes to immunosuppression and maintenance of immunological tolerance by enhancing conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ regulatory T cells (for review see Li *et al.*, 2006). Numerous studies investigated the effects of TGF-βI on mast cells. This cytokine stimulates the chemotaxis of human mast cells (Olsson *et al.*, 2000), influences the differentiation and functions of isolated human intestinal mast cells (Gebhardt *et al.*, 2005), and downmodulates FcεRI expression (Gomez *et al.*, 2005). After a few day long treatment TGF-βI can induce mast cell apoptosis (Norozian *et al.*, 2006). Furthermore, TGF-βI seems to be essential for the maturation of *in vitro*-differentiated mouse mucosal mast cells (Miller *et al.*, 1999).

TGF-βI is thought to have a dual role in cancer progression. The elevated incidence of chemically induced or spontaneously occurring tumors in hemizygous or homozygous *Tgfb-1*-null mice and the decreased risk of cancer in TGF-βI-overexpressing animals clearly demonstrate the tumor-suppressive effects of this cytokine (Akhurst and Derynck, 2001; Derynck *et al.*, 2001; Wakefield and Roberts, 2002). However, many tumors are also able to produce large quantities of TGF-βs and during tumorigenesis apoptotic and

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Abbreviations: GDF-15, growth differentiation factor-15; MC(T), tryptase-containing mast cell; MC(TC), tryptase and chymase-containing mast cell; PBS, phosphate-buffered saline; TGF, transforming growth factor; TIM-3, T-cell Ig and mucin domain-containing protein 3

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growth-inhibitory effects of TGF-βs are lost. Different cancer types, such as melanomas downregulate or modulate specific steps of TGF-β-induced signal transduction pathways that leads to the unresponsiveness of tumor cells to this cytokine (Markowitz and Roberts, 1996). Melanomas are also able to constitutively produce TGF-βI and melanoma progression correlates with the overexpression of TGF-β growth factors (Moretti *et al.*, 1997). As TGF-β concentration is getting higher, tumor cells acquire a more migratory and invasive phenotype. TGF-β can reach this oncogenic and invasion-supporting effect through local immunosuppression, stimulation of tumor angiogenesis, or by altering stromal environment.

Many studies investigated the immune response against tumor cells and tried to identify key molecules that modulate antitumor immunity. In these attempts TIM-3 may have an outstanding role as it has been described to be Th1 cell and $CD8^+$ T cytotoxic 1 cell-specific protein, and Th1 cells express TIM-3 after two or three rounds of stimulation under Th1-polarizing conditions (Monney *et al.*, 2002). TIM-3 has been suggested to be a negative regulator of immune responses with limiting the expansion of activated Th1 and T cytotoxic 1 cell populations and was thought to be highly specific for the above-mentioned cells. Furthermore, TIM-3 has been reported to inhibit the auto- and alloimmune responses and to promote immunological tolerance in a diabetes model system (Sanchez-Fueyo *et al.*, 2003). Despite the increasing number of published reports about TIM-3 in the mouse, its role is still not fully understood and only a limited amount of human data is available.

An elevated number of mast cells has been observed in or around tumorous tissues, and these cells can belong to either the MC(TC) or the MC(T) type depending on the local environment (Ibaraki *et al.*, 2005, Rojas *et al.*, 2005), but there is still an intensive debate if they are beneficial or detrimental for tumor cells. In melanomas, mast cells are thought to contribute to tumor development and progression by causing UV-B-induced immunosuppression (Grimbaldeston *et al.*, 2004; Ch'ng *et al.*, 2006) after degranulation and TNF-α and histamine secretion. This latter molecule has also been shown to enhance melanoma cell proliferation (Hegyesi *et al.*, 2001). Furthermore, mast cells are major sources of potential angiogenic factors, such as fibroblast growth factor-2 or vascular endothelial growth factor (for review see Ch'ng *et al.*, 2006). Usually the dominance of MC(TC) in tumors over the MC(T) population is a predictor of good prognosis in some types of adenocarcinomas or in renal tumors (Beil *et al.*, 1998; Nagata *et al.*, 2003). Based on the high level of TGF-βs in different tumors, such as in melanomas, we assumed that this cytokine can cause gene expression changes influencing interactions between mast cells and tumor cells. As our mast cell cultures did not contain chymase either in the presence or absence of TGF-βI, our experiments were carried out in MC(T) cells. Microarray experiments showed that only 45 genes out of the >40,000 spotted on the chip responded to TGF-βI treatment. The downregulation of growth differentiation factor (GDF)-15 and upregulation of T-cell Ig and mucin domain-containing protein (TIM)-3 in mast cells is indicative for the tumor growth enhancing activity of TGF-β-treated mast cells. Interestingly, the majority of TIM-3 ligand galectin-9 derives not from tumor cells, but rather from mast cells. This may result in an autocrine mechanism leading to immunosuppression through CD80-CTLA4. TIM-3 expression was detected not only in *in vitro*-differentiated, but in melanoma-surrounding mast cells, too. Furthermore, contradicting to the Th1-specificity of TIM-3, this molecule is also expressed in melanoma cells and cell lines at a higher level than in normal melanocytes. The elevated expression of TIM-3 in melanoma cells may be responsible for their lower adhering capacity and thus it can contribute to tumorigenesis.

RESULTS

Characterization of mast cell cultures

Mast cells differentiated for 5 weeks in culture were magnetically separated and then further cultured for an additional 5 days in the presence or absence of TGF-βI. Real-time PCR showed that human mast cells expressed both components (TGF-βRI and TGF-βRII) of the functional receptor for TGF-βI (Figure 1a). Furthermore, TGF-βI did not influence either the cell number determined by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assay or cell viability according to Trypane blue staining (data not shown). To be sure about the effectiveness of TGF-βI-treatment, c-kit (CD117) expression was studied by flow cytometry as downmodulation by TGF-β of this cell surface receptor has been published (Gebhardt *et al.*, 2005). Figure 1b shows a marked reduction in CD117 expression of treated mast cells. Furthermore, no morphological alterations between untreated and treated human mast cells were seen with toluidine blue staining (Figure S1).

The most characteristic difference between human mast cell populations is their protease content. As flow cytometric analysis showed that mast cells cultured in the presence or absence of 2 ng/ml recombinant human TGF-βI were uniformly positive for tryptase (Figure 2a), but they did not

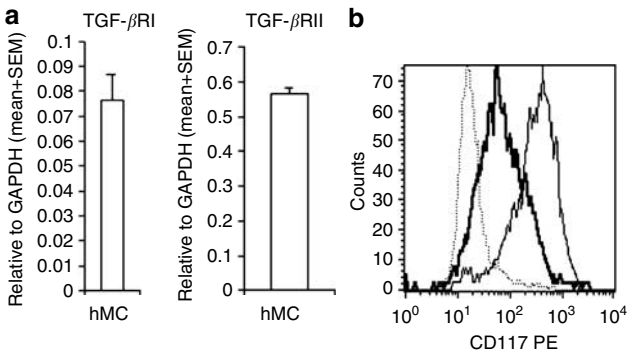

Figure 1. Cord blood-derived mast cells magnetically isolated after 5-week culture expressed TGF-βRI and TGF-βRII and they responded to TGF-βI. (**a**) TGF-βRI and TGF-βRII expression in cultured human cord blood-derived mast cells (real-time PCR). (**b**) Flow cytometry with anti-human CD117 PE. Dotted line: isotype control; simple line: untreated mast cells; bold line: mast cells treated with 2 ng/ml TGF-βI for 5 days.

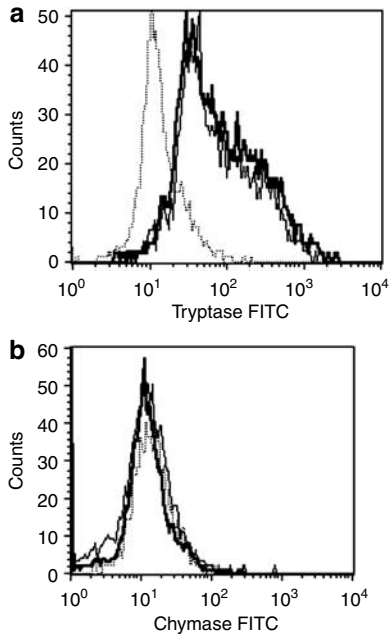

Figure 2. The mast cell cultures contained only MC(T) cells. Both control and TGF-βI-treated (2 ng/ml, 5 days after 5 weeks culturing) human mast cell cultures expressed (**a**) tryptase, but none of them were positive for (**b**) chymase (flow cytometry). The figure shows one representative out of the four repeated experiments. Dotted line: isotype control; simple line: untreated mast cells; bold line: treated mast cells.

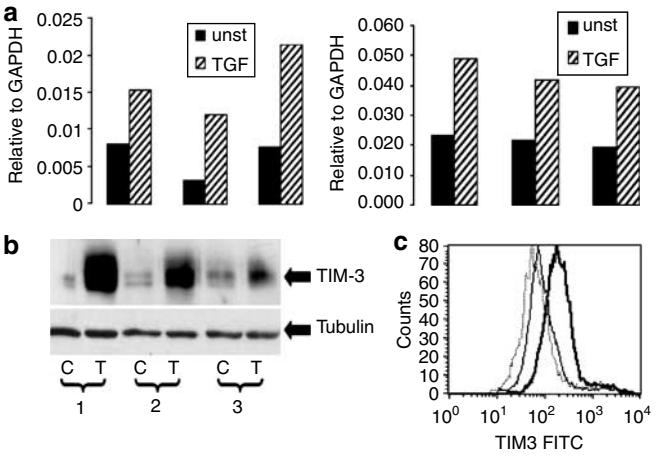

Figure 3. TGF-βI upregulates TIM-3 in human mast cells. (**a**) Real-time PCR results for TIM-3 from human mast cells with TaqMan probes fitting to either exon6/7 (right, $P=0.007$) or exon 2/3 (left, $P=0.025$). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase housekeeping (three paired samples are shown, the *P*-values were determined by paired *t*-test). (**b**) TIM-3 is overexpressed in human mast cells by TGF-βI (2 ng/ml, 5 days) as detected by immunoblot (with tubulin housekeeping). C: control; T: TGF-βI-treated samples; 1,2, and 3: different mast cell preparates. (**c**) TIM-3 level is increased on the surface of human mast cells (flow cytometry) after TGF-βI treatment (2 ng/ml, 5 days). Dotted line: isotype control; simple line: untreated, bold line: treated mast cell preparate.

express chymase at all (Figure 2b), we concluded that our mast cells belonged to the MC(T) group. The presence of only MC(T) cells in our culture is further supported by the fact that cathepsin G, another MC(TC)-characteristic protease was not detected by Western blotting (data not shown).

Microarray measurements

RNA samples derived from five TGF-βI-treated and control cultures were compared by DNA microarray measurements with one dye-swap replicate. According to our analysis, genes upregulated in some hybridizations but downregulated in other ones (anticorrelated genes) were not observed when comparing the chips pairwise, which means that the expression changes are characteristic for the treatment and not for the individual samples. The error-weighted Pearson's correlation coefficients between microarrays were always >0.85 confirming that microarray measurements showed good correlation with each other.

Surprisingly, out of the >40,000 investigated sequences only 45 genes showed a differential expression (>2.0-fold change) at $P<0.05$ significance level (with Benjamini and Hochberg false discovery rate multiple testing correction). The up- and downregulated genes are listed in Table S2.

Gene groups based on gene ontology

Differentially expressed genes were also clustered according to gene ontology cellular component categorization. Table S3 shows that membrane category in TGF-β-influenced gene list is strikingly over-represented.

Validating microarray results

Expression changes of selected genes were validated either by real-time PCR and/or at protein level. For example, the hyaluronic acid receptor CD44 was shown to be upregulated by TGF-β (flow cytometry: the median of fluorescence increased by 112, 46, and 51% at three different preparates) and there was a clear repression of the TGF-β family member GDF-15 as validated by Western blotting (the corrected lane density decreased by 39, 60, and 63% at three mast cell preparates).

TIM-3 is expressed in human mast cells and is upregulated by TGF-βI

One of the most strikingly upregulated genes after TGF-β-treatment is hepatitis A virus cellular receptor 2 or TIM-3 that was originally discovered as a Th1-specific protein. Both probes fitting to exon7 (the last exon of TIM-3 gene) in Agilent Whole Human Genome probe set showed differential expression in our microarray experiments. According to public databases (GeneBank, Ensembl, Swissprot) not only the murine (Sabatos *et al.*, 2003), but the human TIM-3 gene also encodes an alternatively spliced form. Beside the full-length transmembrane protein a secreted one is also produced by using only exon1 and exon2. To investigate the expression of the full-length messenger RNA only, real-time PCR experiments with TaqMan probes fitting to either exon2/3 or exon 6/7 were carried out and results confirmed the upregulation of TIM-3 gene (Figure 3a). By using an antibody against the extracellular domains of TIM-3 we could find different protein levels between control and TGF-β-treated human mast cells with Western blotting (Figure 3b)

and flow cytometry showed the increased cell surface TIM-3 expression when adding TGF-βI to the culture medium (Figure 3c).

TIM-3 is expressed in melanoma-surrounding human mast cells and in melanoma cells

As mast cells are often accumulated in or around melanomas and other tumors and MC(T) cells are supposed to be beneficial for tumor cells, TIM-3 expression was studied in tissue sections from three different patients. Immunohistochemistry revealed that both melanoma cells (Figure 4a) and melanoma-surrounding mast cells (Figure 4b) showed a clear positivity for TIM-3. Mast cells were identified in serial sections with toluidine blue based on metachromatic staining (Figure 4b). The presence of TIM-3 was also confirmed by real-time PCR in WM35 and HT168-M1 melanoma cell lines, and no expression difference was found either between the cell lines or between TaqMan assays fitting to exon 6/7 or exon 2/3 (Figure 4c). In addition, TIM-3 was detected in both WM35 and HT168-M1 with flow cytometry (Figure 4d) and its expression was higher than in HEMn-DP human neonatal epidermal melanocytes (Figure 4e). Furthermore, TGF-βI did not modify either the messenger RNA or cell surface TIM-3 level in melanoma cells (data not shown).

The TIM-3 ligand galectin-9 is expressed in human mast cells

Recently galectin-9 has been identified as the ligand for TIM-3. To determine the potential sources of galectin-9 in melanomas, WM35, HT168-M1 cell lines and human mast cells were studied by real-time PCR for TIM-3 ligand expression. Interestingly, an about ~3-fold order of magnitude higher level of galectin-9 was detected in mast cells compared to melanoma cell lines (Figure 5). Furthermore, TGF-βI did not significantly modify galectin-9 expression in mast cells (paired *t*-test: $P > 0.05$, data not shown).

DISCUSSION

Human $CD34^+/CD117^+/CD13^+$ mast cell precursors (Kirshenbaum *et al.*, 1999) enter from bone marrow into the blood and then migrate to peripherial tissues where they mature under local influences of cytokines, for example of TGF-β. As many tumors, such as melanomas are capable of producing TGF-βI at high level, furthermore, cancer cells become resistant to the apoptotic effect of this cytokine and TGF-β has a clear immunosuppressive role stimulating tumor progression and metastasis formation in later stages of cancer development, therefore we investigated gene expression changes of cord blood-derived, *in vitro*-differentiated human mast cells after TGF-βI treatment by DNA microarray. TGF-βI has been published to induce apoptosis both in human and murine mast cells (Norozian *et al.*, 2006) after some days. To avoid the apoptotic effect, we applied a short treatment with a low TGF-βI concentration.

The ratio of MC(T) and MC(TC) cell types during *in vitro* differentiation largely depends on the exact culture conditions. For example, the presence of high level stem cell factor prefers the development of chymase-positive cells, whereas stem cell factor at a concentration of about 50 ng/ml supports the appearance of MC(T) cells (Ahn *et al.*, 2000). Bagga *et al.* (2004) reported that lysophosphatidic acid accelerates human mast cell development and chymase-positive cells were not found under such culture conditions. As MC(T) cells are supposed to be beneficial for tumor cells, we focused on this cell type and applied stem cell factor at 40 ng/ml with lysophosphatidic acid in our differentiation experiments. According to flow cytometric measurements our cultures did not contain chymase, but expressed tryptase at high level, suggesting the appearance of only MC(T) cells. This conclusion is further supported by the fact that cathepsin G, another MC(TC)-specific protease could not be detected by Western blotting.

Mast cells are capable of responding to a variety of external stimuli. After TGF-β treatment, many differentially regulated genes fall into the membrane category (containing those surface molecules that influence cell–cell or cell–extracellular matrix interaction) according to gene ontology cellular component classification. The hyaluronic acid receptor (CD44) is well known in the human HMC-1 mast cell line (Kruger-Krasagakes *et al.*, 1996), in mast cells isolated from different sources (Beil *et al.*, 1998; Ghannadan *et al.*, 1998) and in cord blood-derived mast cells (Fukui *et al.*, 2000). The upregulation of CD44 in TGF-βI-treated human mast cells suggests that the extracellular matrix component hyaluronic acid may have an outstanding role in the tissue localization of MC(T) cells in tumors, and in the more vigorous interaction between MC(T) cells and the extracellular matrix in cancers.

Our microarray analysis shed light on one differentially regulated secreted molecule, GDF-15/macrophage inhibitory cytokine-1 that is a divergent TGF-β family member. GDF-15/macrophage inhibitory cytokine-1 inhibits proliferation of breast (Li *et al.*, 2000) and prostate carcinoma cells (Tan *et al.*, 2000). GDF-15 overexpression in HCT-116 colon cancer cells resulted in reduced tumor growth in nude mice (Baek *et al.*, 2001). These data suggest that GDF-15/macrophage inhibitory cytokine-1 may function as a tumor suppressor at least in some cancer types. Although no published results are available about the role and expression of GDF-15 in melanomas, based on its downregulation in human mast cells under the influence of TGF-βI, it is conceivable that MC(T) cells enhance melanoma progression partly by this mechanism.

One of our most striking findings is the presence of hepatitis A virus cellular receptor 2 or TIM-3 in human mast cells and its upregulation following TGF-β treatment. This molecule was thought to be highly specific for activated Th1 and T cytotoxic 1 cells, however, further studies revealed the expression of TIM-3 in other cells types, too. Khademi *et al.* (2004) detected TIM-3 messenger RNA in natural killer T cells, Gielen *et al.* (2005) proved its expression in the resident cells of the nervous system, and in our study we provide evidence for the presence of TIM-3 in human mast cells. The ligand for TIM-3 has recently been identified as galectin-9, a galactose-binding lectin that induces apoptosis and necrosis in activated Th1 lymphocytes (Zhu *et al.*, 2005). Binding sites for TIM-3 (TIM3L) have been detected on the

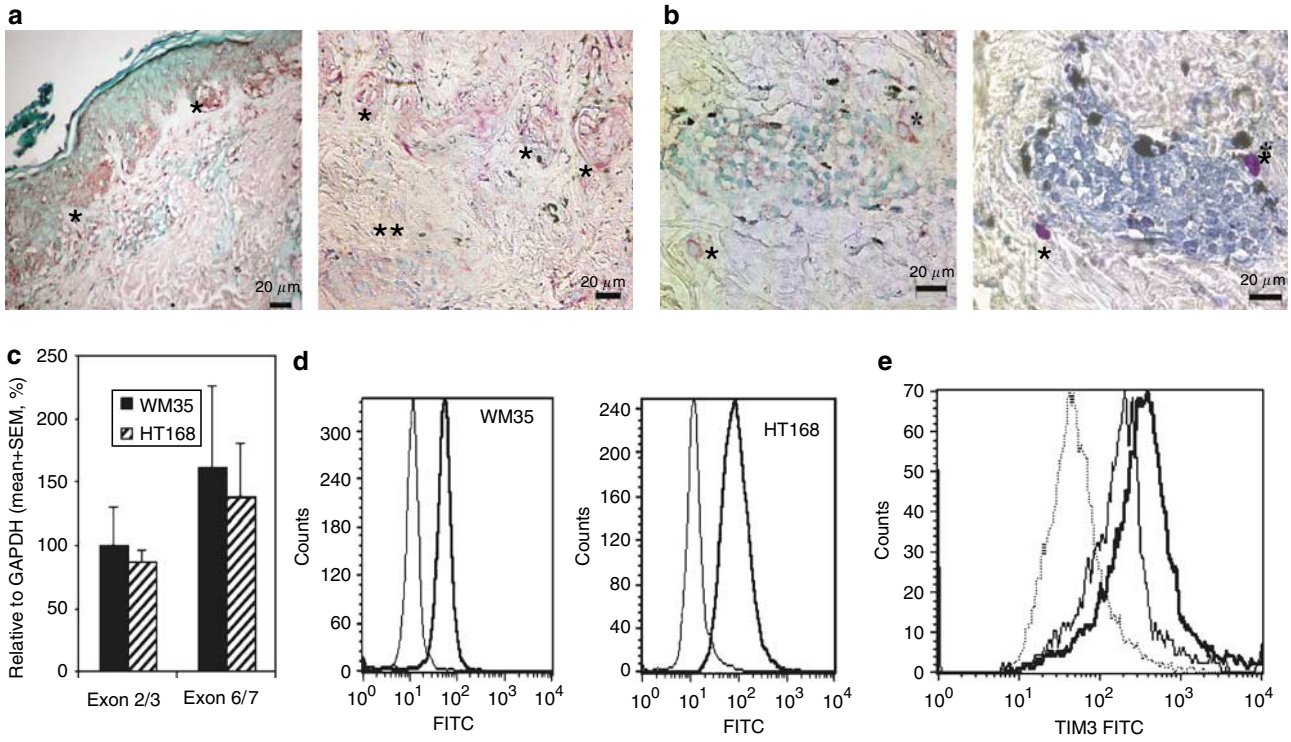

Figure 4. TIM-3 is expressed both in melanoma-surrounding human mast cells and in melanoma cells. (**a**) Immunohistochemistry with anti-TIM3 antibody in melanoma tissue sections. Two representatives out of three different patients. The background staining of the control slides was negligible (see Materials and Methods). Single asterisks indicate melanoma cells. Double asterisk labels the area that is magnified in (**b**). Bar = 20 μm. (**b**) Asterisks mark mast cells in serial sections. Left side: immunohistochemistry with anti-TIM3 antibody (the magnification from a part of **a**). Right side: mast cells show metachromatic staining with toluidine blue. Bar = 20 μm. (**c**) WM35 and HT168-M1 melanoma cell lines express TIM-3 as detected by real-time PCR with TaqMan probes fitting to either exon6/7 or exon2/3 (mean + SEM, $n = 4$, the WM35 data obtained by TaqMan assay fitting to exon 2/3 were taken as 100%). (**d**) Both WM35 and HT168-M1 melanoma cell lines were positive for cell surface TIM-3 (flow cytometric analysis). Simple line: isotype control, bold line: anti-TIM3. (**e**) Melanoma cell lines express TIM-3 at a higher level than HEMn-DP melanocytes (flow cytometry, simple line: melanocytes; bold line: WM35; dotted line: isotype control. With HT168-M1 similar results were obtained).

surface of many cell types, such as on naïve $CD4^+$ T cells, macrophages, dendritic cells (Sabatos *et al.*, 2003), and $CD4^+CD25^+$ regulatory T cells (Sanchez-Fueyo *et al.*, 2003). Anti-TIM-3 antibody decreased TIM-3 and CD80 expression on murine mast cells during acute myocarditis and reduced Treg populations (Frisancho-Kiss *et al.*, 2006). Based on these known data we suggest that TGF-β-stimulated mast cells may support the progression of tumor partly through the higher TIM-3 expression. The higher TIM-3 level may result in an elevated CD80 expression of human mast cells and CD80 can interact preferentially with the inhibitory molecule CTLA-4 leading to local immunosuppression. Indeed, previous studies have reported that blocking CD80 results in increased $CD4^+$ T-cell proliferation and impaired Treg activity (Zheng *et al.*, 2004) and inhibiting CTLA-4 function leads to a more severe diabetes and other organ-specific autoimmune diseases.

Interestingly, TIM-3 is expressed not only by mast cells around melanomas, but by tumor cells in tissue sections, too. Furthermore, human melanoma cell lines WM35 and HT168-M1 were also positive for TIM-3. Dramatically increased levels of the oncogenic protein SKI have been reported in melanomas that represses the TGF-β-initialized signal-transduction pathway (Medrano, 2003), therefore it was not surprising that TGF-β did not modify the expression of TIM-3. The presence of TIM-3 on melanoma cells can explain some known effects of galectin-9 in tumors. According to published studies galectin-9 induces aggregation and reduces the adhesion of breast tumor cells to extracellular matrix proteins (Irie *et al.*, 2005) and similar results were published in melanoma cells. Interestingly, the aggregation of melanoma cells was observed at a concentration of about 10^{-8} M, but it becomes apoptotic only at an order of magnitude higher level (Kageshita *et al.*, 2002). Although the extracellular galectin-9 concentration in melanomas is not known, it probable does not reach such a high level to be able to induce apoptosis in tumors. Furthermore, TIM-3 level is higher in melanoma cell lines compared to normal epidermal melanocytes, which may result in an enhanced sensitivity to galectin-9 and this may explain the reduced adhering capacity of tumors.

To investigate the possible sources of endogenous galectin-9 in melanomas, real-time PCR was carried out in human mast cells and melanoma cell lines. Interestingly, mast cells expressed galectin-9 at about 1,000 times higher level than melanoma cell lines suggesting that the majority of this protein derives not from tumor cells. In support of our

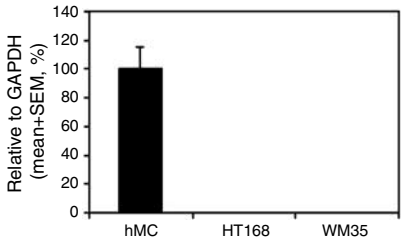

Figure 5. Galectin-9 messenger RNA level is about 1,000-fold greater in human mast cells than in melanoma cell lines. Real-time PCR results were normalized to glyceraldehyde-3-phosphate dehydrogenase housekeeping (mean + SEM, $n = 4$, Student's *t*-test: $P < 0.001$ for both mast cell WM35 and mast cell HT108-M1 comparisons) and mast cell mean expression level was taken as 100%.

findings galectin-9 has been detected on the surface of HMC-1 human mast cell line (Chabot *et al.*, 2002). In addition, this protein is expressed in the cytoplasm of different melanoma cell lines at a low level, and only a minority of cells was positive for surface galectin-9 (Kageshita *et al.*, 2002). Beside mast cells, galectin-9 was found on Jurkat T-cells (Chabot *et al.*, 2002), furthermore, the presence of TIM-3 ligand on regulatory T cells has been proven. Taken together, the major source of cell surface or secreted galectin-9 does not seem to be tumor cells, but rather mast cells and different T-cell populations. Galectin-9 on mast cells may have an autocrine effect and may enhance the expression of CD80 through TIM-3. Furthermore, TGF-βI seems to regulate not the galectin-9, but the TIM-3 level in mast cells, thus leading to immunosuppression.

However, the gene locus for TIM-3 encodes not only a transmembrane protein, but a soluble isoform lacking the transmembrane domain can be expressed by alternative splicing both in the murine (Sabatos *et al.*, 2003) and in the human system (public databases), too. Initially it was hypothesized that the soluble form binds to TIM-3 ligand and prevents the interaction between TIM-3 and TIM-3 ligand and thus Th1 cells continue to proliferate and to perform effector functions (Meyers *et al.*, 2005). However, Geng *et al.* (2006) have recently found just the opposite, as soluble TIM-3 inhibited T-cell-mediated immune responses and significantly impaired T-cell antitumor immunity in a murine model. In our experiments we focused on the full-length form of TIM-3 by applying TaqMan probes fitting to the full-length TIM-3-specific exons and the soluble form was not investigated. As soluble TIM-3 has been shown to bind to TIM-3 ligand on $CD4^+$ T cells and thus it can suppress the antitumor immunity (Sabatos *et al.*, 2003), the elevated number of full-length TIM-3 molecules on mast cells can also exert a similar effect.

In conclusion, we provide evidence that TGF-βI influences the expression level of 45 genes in human MC(T) cells, among them of CD44, GDF-15, and TIM-3. All these expression changes in human mast cells may contribute to tumor progression enhanced by cancer cell-derived TGF-βI. The downregulation of GDF-15 and the upregulation of TIM-3 in mast cells may result in an immunosuppression in tumors. Based on our findings the major sources of TIM-3 ligand galectin-9 are not the tumor cells. Galectin-9 produced by mast cells may act through TIM-3 in an autocrine way and the upregulation of TIM-3 by TGF-βI may repress the local immune response against tumor cells. Furthermore, the higher TIM-3 expression in melanoma cells compared to epidermal melanocytes may partly be responsible for the reduced adhesion properties of cancer cells.

MATERIALS AND METHODS

Human mast cell cultures

Mononuclear cells from cord blood samples (taken with permission of the National Science Ethical Committee) were separated on Ficoll-Hypaque (Sigma, St Louis, MO) and then $CD34^+$ stem cells were magnetically isolated according to the manufacturer (direct $CD34^+$ progenitor cell isolation kit, Miltenyi Biotech, Germany). Cells were cultured in DMEM medium (Sigma) containing 10% fetal calf serum (Gibco BRL, Paisley, Scotland), penicillin and streptomycin (Sigma), MEM non-essential amino acids (Sigma), 1 mM pyruvate (Sigma), 40 ng/ml stem cell factor, 20 ng/ml IL-6 (Serotec, Oxford, UK) and 3 μM lysophosphatidic acid (Sigma) as it has been reported to accelerate mast cell development (Bagga *et al.*, 2004). Half of the medium was replaced in every 4–6 days. After 5–6 weeks, $CD117^+$ mast cells were separated with mouse anti-human CD117 antibody (Pharmingen, San Diego, CA) and magnetically labeled goat anti-mouse IgG (Miltenyi Biotech, Germany) and further cultured for 5 days in the presence or absence of 2 ng/ml recombinant human TGF-βI (Serotec, Oxford, UK).

Melanoma and melanocyte cell cultures

The WM35 and HT168-M1 human melanoma cell lines were maintained in Rosewell Park Memorial Institute-1640 medium (Sigma) supplemented with 10% fetal calf serum and 0.6 g/l gentamycin (Chinoin Co., Budapest, Hungary). DEMn-DP neonatal human epidermal melanocytes were purchased from Cascade Biologics (Portland, OR) and maintained in medium provided by the manufacturer.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues of superficial-spreading melanomas ($n = 3$; Clark Level II, T1, N0, M0) were cut and mounted onto SuperFrost slides. Specimens were deparaffinated and endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 30 minutes at room temperature. After rinsing in phosphate-buffered saline (PBS), the aspecific binding sites were blocked by normal goat serum (Dako, Denmark) for 60 minutes at room temperature and all specimens were incubated with anti-human TIM-3 antibody (4 μg/ml, R&D Systems, Minneapolis, MN) diluted in PBS with 5% BSA, for 60 minutes at room temperature in a humid chamber. As negative control, a specific goat serum was used (Sigma). After washing in PBS, specimens were incubated with biotinylated anti-goat antibody (Sigma) for 30 minutes at room temperature and then washed and incubated with streptavidin/peroxidase complex reagent (Novostain Universal Detection Kit, Novocastra Laboratories Ltd, UK) for 20 minutes. The color was developed by the Vector NovaRED Substrate Kit (Vector Laboratories Inc., Burlingame, CA) and the slides were counterstained with methyl-green (Dako, Denmark). All negative controls demonstrated negligible background fluorescence.

Toluidine blue staining

Small aliquots of mast cell cultures were cytocentrifuged, fixed in ice-cold methanol for 10 minutes and stained with 0.1% Toluidine blue (pH = 1) for 45 minutes at room temperature. Tissue sections were stained in 0.1% Toluidine blue, pH = 4.

1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan assay

Cells cultured in 96-well plates were treated with 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (Sigma) at a final concentration of 0.5 mg/ml. After 4 hours cells were centrifuged, the cell pellet was lysed in DMSO and the absorbance was measured at 540 nm.

Flow cytometry

In case of adherent cells, cells were first removed from tissue culture dishes by 0.2% EDTA (Sigma). Cells were washed twice with PBS, labeled at room temperature for 25 minutes and then analyzed by a FACSCalibur instrument (BD Biosciences, San Jose, CA). The following antibodies were used in our experiments: anti-human CD117 PE, anti-human CD44 FITC (Pharmingen), goat anti-human TIM3 (R&D Systems), mouse anti-human tryptase, mouse anti-human chymase (Chemicon, Temecula, CA), anti-mouse IgG FITC and anti-goat IgG FITC (Sigma). For intracellular FACS analysis, cells were fixed in 2% paraformaldehyde for 20 minutes and permeabilized by 0.1% saponin (Sigma) before labeling.

RNA isolation, quality determination, and microarray measurements

RNA from mast cell samples was prepared by RNAEasy columns (Qiagen, Valencia, CA), the quality and quantity of RNA was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only those samples were used either for microarray or for real-time PCR experiments that gave >8.0 for RNA integrity number, showed a clear gel image and no DNA contamination was observed on the histogram.

200–200 ng total RNA was reverse transcribed by the Low-input RNA Linear Amplification Kit (Agilent Technologies) and then transcribed to either Cy3- or Cy5-labeled cRNA according to the manufacturer. The labeled cRNA was purified (RNAEasy kit, Qiagen, Valencia, CA), the dye content (>8.0 pmol dye/μg cRNA) and the concentration of cRNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of Cy3 and Cy5-labeled cRNA were mixed and hybridized to Agilent Whole Human Genome Oligonucleotide microarrays overnight at 60°C, washed by the ozone-safe saline-sodium phosphate EDTA (SSPE) buffer method, the slides were treated with Stabilizing and Drying Solution (Agilent Technologies) and scanned by Agilent Microarray Scanner. All the steps were carried out according to the manufacturer (Agilent Technologies). Data were then normalized by the Feature Extraction software version 7.5 with default parameter settings for oligonucleotide microarrays and then transferred to the Rosetta Luminator (Rosetta Biosoftware, Seattle, WA) and GeneSpring 7.3 program (Agilent Technologies) for further statistical evaluation. To ensure that only high-quality DNA microarray data are analyzed, some spots were randomly selected and the Cy3- and Cy5 intensity across these spots was analyzed. Furthermore, the distribution of up- and down-regulated genes across the microarrays was also evaluated. In GeneSpring the boxplot view was used for quality control and those microarrays passed that had the median log ratio near to zero after normalization steps recommended by Agilent Technologies for two-color data (but the per gene step was omitted).

In our microarray experiments five biological and one technical (dye-swap) replicates were included. Experiments were built from these microarrays by GeneSpring and those genes were further evaluated that showed a >2.0-fold change. This gene list was then filtered for *t*-test *P*-value ($P<0.05$ and the Benjamini and Hochberg false discovery rate correction was used for multiple testing). All our microarray data have been submitted to the GEO database (www.ncbi.nlm.nih.gov/geo/) under the series excession number GSE4906.

Real-time PCR

1 μg quality-checked RNA was reverse transcribed by 1U murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) with random primers (Promega, Madison, WI) at 42°C for 55 minutes. Murine leukemia virus was then inactivated at 95°C for 5 minutes. Real-time PCR reactions were carried out in an ABIPrism 7000 instrument according to the manufacturer (Applied Biosystems) with 1.5 μl cDNA in each well and in 25 μl final volume. The applied TaqMan gene expression assays are listed in Table S1.

Immunoblotting

Cells were denatured in lysis buffer (100 mM Tris, 1% Triton X-100, 1 mM EGTA, 2.5 mM NaF, 10 μg/ml leupeptin, 2 mM phenylmethylsulphonyl fluoride, 10 mM Na-vanadate, 1% aprotinin) and protein concentration was determined with the Bradford method. Protein (15 μg) from whole-cell lysate/sample was denatured and reduced in β-mercaptoethanol at 100°C for 5 minutes, applied to 12.5% SDS-PAGE and then transferred to P-Immobilon (Millipore, Billerica, MA) membrane. The full-range Rainbow (Amersham Biosciences, Piscataway, NJ) was used as molecular weight marker.

Blots were blocked with 10% milk powder in PBS for 1 hour and then incubated with the primary antibody in PBS containing 1% milk powder and 0.1% Tween-20 for 2 hours at room temperature. After excessive washings horseradish peroxidase-conjugated secondary antibodies were applied for 45 minutes and the signals were developed by ECL Plus Western Blotting Detection System (Amersham Biosciences). To check the loaded protein quantity, tubulin was measured as housekeeping. The following antibodies were used: goat anti-human cathepsin G (0.5 μg/ml), goat anti-human Tim3 (0.5 μg/ml), goat anti-human GDF15 (0.5 μg/ml) (R&D Systems), rat anti-tubulin (0.5 μg/ml, Serotec), anti-rat horseradish peroxidase (1:10,000), anti-goat horseradish peroxidase (1:8,000, Sigma). The density of specific lanes was determined by ImageQuant software version 3.3 and these values were devided by tubulin housekeeping signals (the local background was always subtracted).

Statistical analysis

Student unpaired or paired *t*-tests were applied to analyze the statistical significance of our results.

Statements

All experiments were carried out according to the Declaration of Helsinki Principles and with permission of the Hungarian Science

Ethical Committee. Before the experiments written informed consent was signed by the cord blood donors.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. TaqMan gene expression assays used in our studies (Applied Biosystems, Foster City, CA).

Table S2. Genes differentially regulated (>2.0-fold change, $P<0.05$ with Benjamini and Hochberg false discovery rate multiple testing correction) between control and TGF-βI-treated human mast cells.

Table S3. Genes listed in Table S2 were classified according to gene ontology cellular component.

Figure S1. Cord blood-derived mast cell praparates magnetically isolated after 5-week culture were >90% pure and TGF-βI (2 ng/ml, 5 days) did not alter cell morphology.

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