Fakulteten för Veterinärmedicin och husdjursvetenskap
Institutionen för anatomi, fysiologi och biokemi

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SUMMARY

Mast cells have for a long time been known to accumulate around tumors (Maltby *et* al., 2009). Studies show that they may be important and sometimes essential in tumor angiogenesis, tumor growth and metastasis of tumors (Soucek, *et al*., 2007; Xiang *et al.*, 2010). In an expression array study (Wensman *et al*, submitted manuscript) performed on mouse mast cells exposed to tumor conditioned medium, the gene 4-1BB was among the most up-regulated genes compared to control medium. 4-1BB codes for a membrane receptor protein of the tumor necrosis factor superfamily and has been shown to be important in T cell regulation in tumor diseases. This study was performed to investigate if human mast cells up-regulate 4-1BB when they get exposed to tumor conditioned medium. Two human mast cell lines were used: HMC-1 and Lad-2. When comparing them, Lad-2 had a more clear response to tumor conditioned medium and showed higher expression of 4-1BB. Two different tumor cell lines were used for studying the mast cell response to tumors: The thyroid carcinoma cell line Kat-4 and the melanoma cell line SK-MEL-30. Using Kat-4 to stimulate the human mast cells led to an up-regulated gene expression of 4-1BB while the use of SK-MEL-30 as a stimulant did not. The gene expression of 4-1BB in Lad-2 peaked after four hours of stimulation with serum free medium conditioned with Kat-4 cells. After twenty-four hours the gene expression of 4-1BB decreased, which is a pattern that followed what Nishimoto *Et al*. showed in 2005 where expression of 4-1BB was investigated in mouse mast cells. Protein expression of 4-1BB was measured after zero, four and twenty-four hours. No up-regulated protein expression of 4-1BB was observed in neither HMC-1 cells nor Lad-2 cells after stimulation with tumor cell line conditioned medium, however there was an increase of 4-1BB protein in response to calcium ionophore. In this study human mast cells responded to stimulation of tumor factors by significantly up-regulate the gene 4-1BB. Further, a correlation between expression of 4-1BB mRNA and 4-1BB protein was not observed.

SAMMANFATTNING

Det har länge varit känt att mastceller ansamlas runt tumörer (Maltby *et* al., 2009). Studier visar att de kan vara viktiga och ibland nödvändiga för kärlnybildning, tumörtillväxt och tumörspridning (Soucek, *et al*., 2007; Xiang *et al.*, 2010). Vid ett experiment (Wensman *et al*, insänt manuskript) med mastceller från mus som inkuberades i tumörstimulerat medium visade sig 4-1BB vara en av de gener som uppreglerades mest jämfört med kontrollmedium. 4-1BB är ett membranprotein i tumörnekrosfaktorsuperfamiljen som kodar för en receptor viktig i t-cellsreglering vid tumörsjukdom. Den här studien syftar till att undersöka huruvida humana mastceller uppreglerar 4-1BB när de exponeras för tumörkonditionerat medium. Två humana mastcellinjer användes: HMC-1 och Lad-2. När de jämfördes visade Lad-2 en tydligare respons på tumörkonditionerat medium och ett högre uttryck av 4-1BB. Två olika tumörcellinjer användes: Thyroideacarcinomcellinjen Kat-4 och melanomcellinjen SK-MEL-30. Genuttrycket av 4-1BB var som högst hos Lad-2-celler efter fyra timmars stimulering i serumfritt medium konditionerat med Kat-4-celler.

Efter tjugofyra timmar sjönk genuttrycket av 4-1BB vilket följer mönstret som bl.a. ses i en studie av Nishimoto *Et al*. från 2005 där uttrycket av 4-1BB hos musmastceller studerades. Proteinuttryck av 4-1BB mättes efter noll, fyra och tjugofyra timmar. Inget uppreglerat proteinutryck av 4-1BB observerades i vare sig HMC-1-celler eller Lad-2-celler efter stimulering med tumörcellinjekonditionerat medium, däremot noterades en ökning av 4-1BB protein som respons på kalciumjonofor. I denna studie svarade humana mastceller på stimulering med faktorer utsöndrade från tumörceller genom att signifikant uppreglera genen 4-1BB. Däremot observerades ingen korrelation mellan uttryck av 4-1BB mRNA och 4-1BB protein.

INTRODUCTION

Mast cells

The mast cell is a type of myeloid cell. Other myeloid cells are erythrocytes, thrombocytes, basophils, neutrophils, eosinophils and macrophages. Mast cells have granules, which contain e.g. histamine and tryptase. Histamine is an amine important in allergic reactions. Tryptase is a protease important in inflammation and anaphylaxis. Mast cells degranulate when they get activated. There are three ways to activate them: by activated complement proteins, cross-linking of Immunoglobulin E (IgE) receptors ($F_{c\varepsilon}RI$) or by direct injury to the cell. Besides being important in allergic reactions and anaphylaxis, mast cells may have an important role in defense against pathogens and tumors (Grewal, 2009).

Mast cells and tumors

Mast cells accumulate around tumors (Maltby *et* al., 2009). For instance, twice as many mast cells per unit area are seen in primary melanomas than in common acquired nevi (moles) (Guidolin *et al.*, 2005). There are also indications that mast cells influence tumor growth (Maltby *et al*., 2009) and that they can either promote (Carlini *et al.,* 2010) or inhibit (Oldford *et al*., 2010) the tumor. Mast cells accumulate in the space between healthy and malignant tissue, the peritumoral area, also called the invasive front, the area of tumor expansion. In breast cancer, mast cell density is higher in peritumoral tissue than in normal breast tissue (Xiang *et al.*, 2010). Mast cells are often found close to blood vessels (Guidolin *et al.*, 2005) and since they express many pro-angiogenic compounds they may be important in tumor angiogenesis (Sawatsubashi *et al*., 2000). Several studies have looked into the correlation between mast cells and vessel count. For example, in lung carcinomas it has been shown that the density of mast cells and micro vessels is higher peritumorally than intratumorally (Carlini *et al.*, 2010). The blood vessel density was almost six times higher in primary melanomas than in common acquired nevi (Guidolin *et al*., 2005).

There are also indications that mast cell and vessel density correlate with the aggressiveness, the grading, of the tumor. In stage IV gastric carcinomas the density of mast cells as well as blood vessels is higher than in stage I, II and III gastric carcinomas (Ribatti *et al.*, 2010). A progressive increase in vessel and mast cell density is seen between stage I and IV. Mast cell density is higher in grade III breast cancers than in grade I and II breast cancers. In breast cancers with lymph node metastasis mast cell density is higher compared to breast cancers without lymph node metastasis. These studies support the theory that mast cells may have a part in tumor angiogenesis and tumor growth. One way for a cell to inhibit tumor growth is by immune activation/suppression. Activated T cells can for example have a part in activity against tumors (Grewal, 2009).

These influences on tumor growth are of great interest from a medical viewpoint. This is mainly because greater insight into the different mechanisms involved may provide knowledge leading to the development of new immunotherapeutic drugs against cancer.

In veterinary medicine mast cells in the context of tumors are generally associated with mastocytomas. However, this study is focusing on mast cells' up-regulation of a specific gene, 4-1BB, after stimulation by a tumor conditioned medium.

Further, *in vivo* experiments have shown that mast cells accumulate around tumors before angiogenesis starts (Crivellato *et al*., 2008). An *in vivo* experiment of epithelial carcinogenesis showed that mast cells might participate in an early stage of tumor development (Coussens *et al*., 1999). Tumor progression correlated with up-regulation of pro-angiogenic coding genes. An in vivo study of pancreatic B-cell tumors showed that fast recruitment of mast cells by activation of Myc (a pleiotropic transcription factor, commonly overexpressed in human tumors, that impacts on for e.g. angiogenesis and remodelling of extracellular matrix) is crucial for tumor growth (Soucek*, et al*., 2007). There are at this point many studies that have addressed the question if mast cells have an effect of the outcome of a tumor disease. Most of the studies have used clinical material and focused on correlations between mast cell densities, location and prognosis. However, so far there have not been so many mechanisms identified and thereby it is relatively unknown how mast cells affect tumor growth and that calls for further studies.

4-1BB

4-1BB belongs to the tumor necrosis factor (TNF) superfamily, a group important in inflammation and immune regulation. TNFs are cytokines produced by e.g. macrophages and T cells. TNF-α is an acute phase protein, which together with other cytokines is important in recruiting immune cells to get a fast immunological response against pathogens. Lymphotoxin, formerly called TNF-β, inhibits tumor growth.

4-1BB is a transmembrane protein expressed on the cell surface (Schwarz, 2005). 4-1BB mRNA was first detected in cytotoxic T cells and T helper cells (Kwon and Weissman, 1989). By confocal laser microscopy the protein was shown to be expressed on the cell surface of cytotoxic T cells and T helper cells (Yunle W et al., 2003). See figure 1. The expression is concentrated to approximately one third of the cell membrane. See figure 1.

Studies have shown that up-regulation of the 4-1BB gene in T cells results in tumor inhibition (Miller *et al.*, 2002; Murillo *et al.*, 2008). Mast cells are also known to express 4-1BB (Nishimoto *et al*., 2005). By binding an agonistic 4-1BB Ab to 4-1BB, a cell can get either stimulated or regulated (Grewal, 2009). Agonistic 4-1BB Ab can stimulate Natural killer cells and T cells. These activated cells may protect against tumors or virus. See figure 2.

When 4-1BB$^{-/-}$ and 4-1BBL$^{-/-}$ mast cells get stimulated through $F_{c\varepsilon}RI$ they have defects in degranulation and cytokine production. Hence, 4-1BB and 4-1BBL may costimulate mast cells as they costimulate T cells, and agonistic 4-1BB Ab may thus be able to stimulate mast cells as they stimulate T cells and Natural killer cells. 4-1BBL, a transmembrane protein (Schwarz, 2005) binding to 4-1BB can activate T cells, which leads to expansion of tumor reactive T cells (Grewal, 2009). It is therefore of interest to investigate the expression of 4-1BB in mast cells when they are exposed to factors secreted by tumor cells. Very little is known about 4-1BB's expression in mast cells. A study performed by Nishimoto *et al.* in 2005 showed that naive mast cells do not express detectable mRNA levels of 4-1BB. After sensitization over night with IgE, 4-1BB was induced. If no more stimulation was done, the levels of 4-1BB decreased during the incubation period. If stimulated with agonistic rat anti-4-1BB monoclonal antibody (mAb) an increase in mRNA-levels of 4-1BB was seen after 8 hours and then the levels decreased over the 48 hours incubation. Protein expression of 4-1BB reached its highest level after 8 hours sensitization with IgE, and after 12 hours the expression decreased.

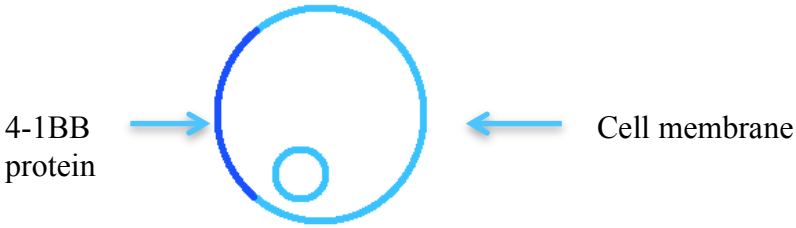

Figure 1. 4-1BB protein is expressed on parts of the cell surface of T cells (Yunle et al., 2003; illustration after Yunle et al., 2003).

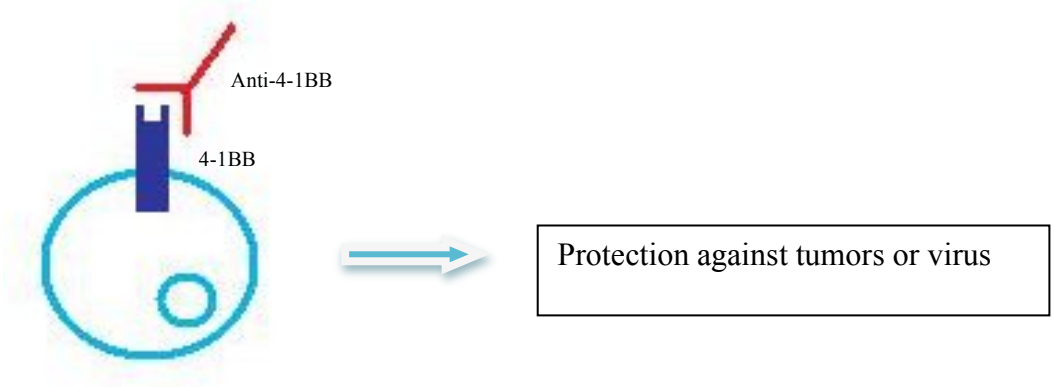

Figure 2. Stimulation of a mast cell by agonistic 4-1BB Ab leads to cell activation and protection against tumors and virus (Grewal, 2009; illustration after Grewal, 2009).

4-1BB and tumor rejection

Treatment with anti-4-1BB in mice with fibrosarcomas often resulted in total tumor rejection or in significant reduction of tumor growth and reduced tumor size (Miller *et al.*, 2002).

Cytotoxic T cells mediated tumor rejection after treatment with anti-4-1BB and this also resulted in long-lasting tumor-specific memory. Anti-4-1BB was used on six tumor models and caused a high rate of rejection in four of them. In mice, anti-4-1BB is effective against HOPC, a myeloma model, NS0 myelomas and 5TGM1, a mouse myeloma cell line reminiscent of human multiple myeloma (Murillo *et al.*, 2008). It gives longer time of survival and a long-lasting anti-tumor immunity. 4-1BB is not expressed on the cell membrane of HOPC, NS0 or 5TGM1. Tumor rejection by anti-4-1BB requires natural killer cells, Cytotoxic T cells and IFN-γ. A phase II clinical trial study, NCT00612664, of anti-4-1BB for treatment of melanomas has recently been carried out, no data has yet been published (U.S. National Institutes of Health [online], 2010). Mice with an established subcutaneous B16 (B16-F10, a melanoma cell line) tumor had a lower rate of tumor growth when treated with Tyrosinase-related protein 2 (pTRP2)-specific allo-restricted cytotoxic T Cells compared to untreated mice or mice treated with naive B6 Cytotoxic T cells (Lu *et al.*, 2008; Lu *et al., 2009*). 4-1BBL is one of the components in pTRP2-specific allo-restricted CTLs. The mice lived longer than the other mice, over 60 days compared to up to 27 days. When B16 tumor cells were given intravenously to provoke the formation of lung metastasis, the group treated with pTRP2-specific allo-restricted CTLs had a lower rate of metastases than the untreated mice, mice treated with naive B6 Cytotoxic T cells or pMut1-specific allo-restricted CTLs (a control peptide). These results indicate that anti-4-1BB can have a part in rejection of tumors or reduction of tumor size. It can also lead to longer time of survival and a long-lasting anti-tumor immunity. This, combined with the phase II clinical trial study on anti-4-1BB, is a strong indicator that anti-4-1BB can be important in immunotherapy against cancer in the future. 4-1BBL may affect tumor growth, tumor metastasis and time of survival in mice with melanomas. It is interesting that it seems to have effects both on subcutaneous tumors and tumor metastasis. If a subcutaneous tumor has a lower rate of growth it can be expected to lead to a longer time of survival and also a lower rate of tumor metastasis. In these studies it is shown that 4-1BBL also may give a direct protection against metastasis when tumor cells are trying to spread through the blood.

Aim of study

The aim of the study was to investigate if human mast cells up-regulate 4-1BB when exposed to tumor conditioned medium. In mouse mast cells exposed to tumor conditioned medium it has been shown that the gene 4-1BB is among the most up-regulated genes and responds with a 13-fold increase of mRNA compared to control medium (Wensman *et al*, submitted manuscript). 4-1BB is known to be involved in defence against tumors, which makes it an interesting gene and that is the main reason 4-1BB was chosen for this study. Today there is not much knowledge about the mechanisms behind mast cells' effect on tumors. In this study we wanted to investigate both gene and protein expression of 4-1BB, in the protein expression we were also interested in the expression pattern.

Study setup

To investigate if mast cells up-regulate 4-1BB when they get exposed to tumors a model for this was used. HMC-1 and Lad-2 cells originating from a human mastocytoma and a mast cell sarcoma/leukemia respectively, served as mast cell models. A study by Guhl *Et al.* in 2010 shows that HMC-1 cells are immature tumor mast cells which makes them very different from peripheral mast cell whereas Lad-2 cells are more differentiated and therefore are more alike peripheral mast cells. Tumor cells secrete a lot of substances to their micro environment to help them expand, infiltrate and metastasize. These substances are thought to influence the mast cells found in or close to the tumor. Medium where tumor cells were cultured, called tumor conditioned medium, contains these substances and when mast cells are incubated in this medium they may up-regulate 4-1BB. Tumor conditioned medium was made by allowing Kat-4, a thyroid carcinoma cell line, or SK-MEL-30, a melanoma cell line, to grow in a fresh medium for two to three days, depending on expansion rate. The media were then filtrated, collected and stored at -20^0 C.

To study if human mast cells up-regulate 4-1BB when exposed to tumor conditioned medium three methods were used. Real time-PCR was used to study gene expression of 4-1BB. Protein expression was studied by two methods: fluorescence activated cell sorting (FACS) and immunohistology. In all three methods the same incubation times were used: zero hours, four hours and twenty-four hours. Samples for RNA and protein expression were taken at the same time, the samples for first and second Real time-PCR were taken when sampling for the FACS experiments and the samples for the third Real time-PCR were collected when sampling for the second immunohistology experiment. In all three methods calcium ionophore was used as a positive control, it activates the mast cell which will express 4-1BB. In immunohistology human spleen was also used as a positive control, which was used since it contains cells that express 4-1BB.

MATERIAL AND METHODS

Cell cultures

The human cell lines used in this study

The HMC-1 cell line was chosen due to their fast proliferation rate. They are however quite unlike peripheral mast cells since they are immature (Guhl *Et al.,* 2010). The cells were cultured in IMDM, from SIGMA, with 10% Fetal Bovine Serum (FBS), 5% 200 mM L-glutamin (GIBCO), 5% PEST (6 mg/ml resp. 5 mg/ml, SVA 992450) and 0,05% α-thioglycerol. The Lad-2 cell line was not used in the early experiments due to the cell's slow proliferation rate and the difficulty in culturing. Lad-2 cells resemble peripheral mast cells since they are more differentiated then HMC-1 cells (Guhl *Et al.,* 2010). The cells were cultured in Stem Pro® - 34 SFM (Serum free medium for Hematopoetic Stem cells), with Nutr sup, GLN and 5% PEST (6 mg/ml resp. 5 mg/ml, SVA 992450).

Kat-4 cells originate from a poorly differentiated human thyroid carcinoma (Husmark *Et. Al.,* 2002). The proliferation rate is high and they show a multi-layered growth. SK-MEL-30 originate from a human melanoma (Romano *et al.*, 2009). This cell line is of interest since mast cells often are found around melanomas (Guidolin *et al.*, 2005). Kat-4 cells and SK-MEL-30 cells were cultured in RPMI 1640 + GlutaMAX^{TM}-I, from GIBCO, with 50% FBS, 5% L-glutamine (GIBCO) and 5% PEST (6 mg/ml respective 5 mg/ml, SVA 992450).

Real time-PCR

RNA was purified from cultured cells with NucleoSpin® RNA II (Macherey-Nagel) according to instructions from the manufacturer. The cells were lysed. To reduce viscosity and to clear the lysate, the samples were filtrated. To adjust RNA binding conditions, 70% RNase-free ethanol was added to the lysates. To bind RNA each mixed lysate was loaded to a column. To desalt and dry silica membranes Membrane Desalting Buffer was added to each column. To digest DNA, DNase reaction buffer (prepared by mixing 10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase (Macherey-Nagel) for each isolation) were applied onto the silica membrane and then the samples were incubated in room temperature for 15 minutes. To wash and dry silica membranes the membranes were washed three times with buffers. To elute RNA, RNase-free water was added to each column.

For synthesis of cDNA from total RNA, as templates, random hexamers (50 µg/µl; Invitrogen) and dNTPs (2 mM) were mixed for each sample. The samples were heated to 65ºC for 5 minutes and then put on ice and chilled for 2 minutes. 5 x strand buffer and 0,1M DTT were added to each sample and then the samples were incubated for 2 minutes at 25ºC. Superscript II (Invitrogen), on ice, was added to each sample and then the samples were incubated for 10 minutes at 25ºC. After that the samples were incubated at 42ºC for 50 minutes and then at 70^0C for 15 minutes.

Real-time PCR, a quantitative PCR, is used to measure amounts of cDNA or mRNA in a sample (Molecular station, 2005-2010). It is commonly used to determine a gene's expression by measuring the amount of mRNA in a sample. It can for example be used for comparing gene expression between control and disease samples.

Primer efficiency for human 4-1BB's primer pair (Integrated DNA Technologies; forward primer: 5´-TGC TTG TGA ATG GGA CGA AGG AGA-3´ and reverse primer: 5´-AGA AAC GGA GCG TGA GGA AGA ACA-3´) was determined from the C_T-values from three different dilutions of cDNA. The C_T-values were plotted for each dilution against log concentration to calculate efficiency, using this equation: 10^(1/slope) 1. A mastermix containing SYBR Mix (Invitrogen), Primer mix (10 μM), referens dye (Invitrogen) and RNase-free water was used. Technical duplicates were used in all experiments. Human GAPDH served as a housekeeping gene. Forward primer: 5´-TCG ACA GTC AGC CGC ATC TTC TTT-3 and reverse primer: 5´-ACC AAA TCG GTT GAC TCC GAC CTT-3´.

The Real time-PCR cycling conditions were 2 minutes at 50^0 C followed by 10 minutes at 95^0C. Then the samples were cycled 40 times at 95^0 C (denaturation) for 15 seconds, at 60^0C (annealing) for 1 minute. ABI Prism 7900 HT using iQ collected data which was analyzed with SDS software. The results were calculated according to User Bulletin #2: ABI PRISM 770 Sequence Detection System (P7N 4303859; Applied Biosystems), a comparative C_T-method.

FACS

Extracellular staining

To reduce unspecific binding, the cells were resuspended in an Fc-block mixture containing 0,5% Fc-block (AbCam) and 3% Bovine serum albumine (BSA)/Phosphate-buffered saline (PBS). Then the samples were incubated on ice for 15 minutes. The cells were either incubated with a mouse anti-4-1BB mAb (AbCam), a mouse IgG polyclonal Antibody ((pAb); AbCam) or 3% BSA/PBS on ice in the dark for 30 minutes. The cells were washed three times with 10% FBS/PBS. Then 10% FBS/PBS was added to each sample. A FACScan (Becton and Dickinson) was used for cell acquisition and Cell Quest software was used for data analyses.

Intracellular staining

The cells were washed with 0,5% BSA/2mM ethylenediaminetetraacetic acid (EDTA)/PBS. Then the cells were resuspended with PBS and 4% formaldehyde and incubated in room temperature for 20 minutes. The cells were washed two times with 1% BSA/0,09% Na azid/PBS. Then the cells were resuspended with 0,5% saponin in 1% BSA/0,09% Na azide/PBS and incubated on ice for 10 minutes. The cells were washed with 1% BSA/0,09% Na azide/PBS two times. To reduce unspecific binding, the cells were resuspended in Fc-block mixture containing 0,5% Fc-block and 99,5% 0,5% BSA/2mM EDTA/PBS. Then the samples were incubated on ice for 15 minutes.

The cells were incubated either with a mouse anti-4-1BB mAb, a mouse IgG pAb or 0,5% BSA/2mM EDTA/PBS on ice in the dark for 30 minutes. The cells were washed two times with 1% BSA/0,09% Na azid/PBS. Then 1% Foramaldehyde/PBS was added to the cells.

Immunohistology

The cytospin specimens were centrifuged for 3 minutes at 800 or 1000 rounds per minute (rpm). After that they were put in room temperature for one hour to dry and then they were stored at -20°C.

Paraffin sections were deparaffinized and rehydrated. The cytospin specimens were fixated in 4% buffered formaldehyde for 10 minutes and then rehydrated. This fixation resembles the fixation used for the paraffin sections. All specimens, paraffin sections and cytospin specimens, were put in a bath with antigen retreival buffer (PT Mudule buffer (Labvision)), and were boiled at 125^0 C for 4 minutes followed by 90^0 C for 4 minutes in a pressure boiler (Pascal, DakoCytomation). They were then washed three times in Tris-buffered saline-Tween 20 (TBS-T). The specimens were covered with a peroxidasblock (Dako) and incubated in room temperature for 5 minutes. They were washed three times with TBS-T. To reduce unspecific binding, goat serum (1:50 or 1:10 + 0,5% BSA) was added to each specimen and they were then incubated in room temperature for one hour. Goat serum was chosen since the secondary antibody was made from goat. The specimens were then incubated with a primary antibody, rabbit anti-4-1BB mAb (AbCam) or rabbit Ig polyclonal antibody (pAb) (isolated from serum, Dako), or TBS-T over night at 4^0C. Rabbit Ig pAb served as negative control since the 4-1BB was a polyclonal rabbit antibody. Mast cells are known to bind antibodies unspecifically and isotype control antibodies are important for the evaluation of the results. If binding pattern of rabbit Ig pAb are compared to binding pattern of rabbit anti-4-1BB mAb the specific binding of rabbit anti-4-1BB mAb can be determined. TBS-T served as negative control to illustrate the specimens without a primary antibody binding to the cells. Paraffin sections from human spleen served as positive controls. Rabbit anti-4-1BB mAb was diluted 1:500 and rabbit Ig pAb 1:1000. The specimens were rinsed with TBS-T and then washed three times with TBS-T. Then peroxidase marked polymer (secondary antibody, Dako) was added to each specimen and they were incubated in room temperature for 30 minutes. After that the specimens were rinsed with TBS-T and then washed three times with TBS-T. Substrate chromogen solution (1% Dab (chromogen)/99% substrate buffer, Dako) was added to each specimen and they were then incubated in room temperature for one minute. The specimens were washed in deionized water and bathed in Mayer (water based hematoxylin) for 30 seconds. The specimens were washed in deionized water and then they were washed for 10 minutes in slowly pouring tap water. Dehydration of the specimens was performed by bathing them in ethanol and xylene. The specimens were mounted with pertex. A Leica DMLA microscope was used and photos were taken with Leica DC 200. For optimizing the photos, Photoshop was used.

RESULTS

To investigate the 4-1BB protein expression in human mast cells exposed to tumor conditioned medium, a FACS experiment with HMC-1 cells was performed. HMC-1 cells were incubated with medium conditioned with Kat-4 cells or control medium. The experiment was performed with cell surface staining. No differences were seen either between conditioned medium and control medium or between the 4-1BB antibody and the isotype control antibody. Figure 3 shows protein expression of 4-1BB and IgG control after twenty-four hours in HMC-1 cells. Since no difference was seen between antibodies or media, a new experiment with the same antibodies but with medium conditioned with SK-MEL-30 cells instead of medium conditioned with Kat-4. The reason for keeping the antibodies is that since they may work it is not wise to change both medium stimulant and antibodies at the same time. The experiment with SK-MEL-30 conditioned medium was also performed with cell surface staining. The result was the same, no differences between conditioned medium and control medium or between the 4-1BB antibody and the isotype control antibody. With this method even a low protein expression could be found. If the mast cells do not express 4-1BB on their cell surface the reason for not finding it can be that they are expressed intracellularly. In 2005 Nishimoto *et al.* showed that mouse mast cells express 4-1BB on their cell surface. It has not been shown that human mast cells express 4-1BB on their cell surface but likely they do. If human mast cells do not express 4-1BB on their cell surface, the protein expression can be found in a FACS experiment with intracellular staining. The next FACS experiment was therefore carried out with intracellular staining. Since Lad-2 cells is a better model for peripheral mast cells than HMC-1 cells they were used instead of HMC-1 cells.

Immunohistology was also used to study human mast cells' protein expression of 4-1BB. By using immunohistology protein expression pattern and localisation of the protein can be observed. In the first immunohistology experiment, HMC-1 cells were either incubated in medium conditioned with Kat-4 cells or control medium. Human spleen served as a positive control. Used antibodies were rabbit anti-4-1BB pAb and rabbit isotype control IgG pAb. TBS-T (no primary antibody) served as a negative control. In human spleen 4-1BB was expressed: there were some strongly positive cells, many moderately positive and some negative cells. No differences were seen between cytospin specimens incubated in medium conditioned with Kat-4 cells for zero and four hours. The rabbit IgG pAb did not work as a negative control since the binding pattern was too unspecific. That made it hard to find the specific expression of 4-1BB. See figure 4. Since HMC-1 cells differ quite much from peripheral mast cells, the next immunohistology experiment was carried out with Lad-2 cells that are more like peripheral mast cells. In the next immunohistology experiment another rabbit Ig pAb was tested but there was not enough time to find the optimal dilution of it so it did not work as a negative control.

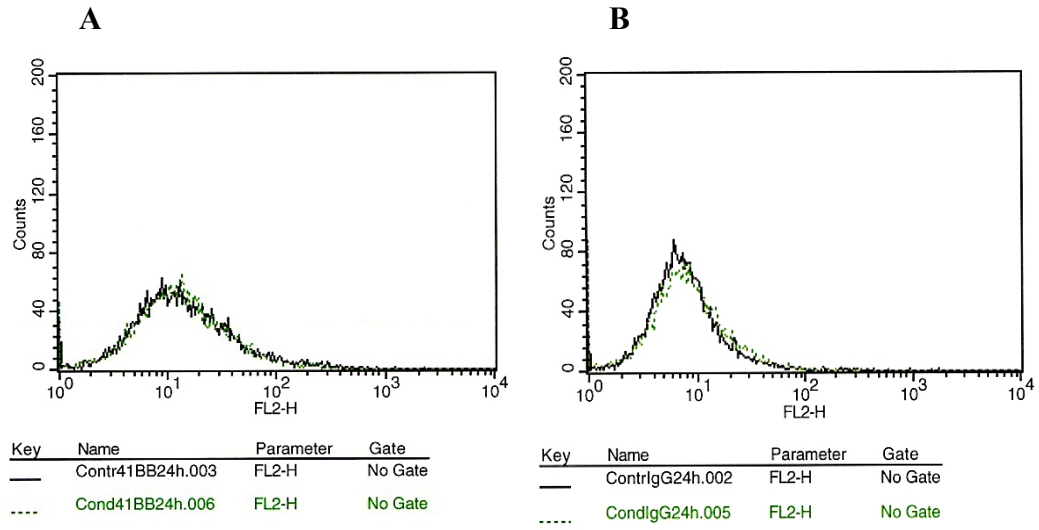

Figure 3. FACS, cell surface staining of HMC-1 cells. A. Expression of 4-1BB in cells incubated for twenty-four hours either in medium with 10% FBS (black) or medium with 10% FBS conditioned with Kat-4 cells (green). No significant difference in expression of 4-1BB was seen between mediums. B. Isotype control antibody binding to cells incubated for twenty-fours hours in medium with 10% FBS (black) and medium with 10% FBS conditioned with Kat-4 cells (green). No significant difference in expression of IgG was seen between mediums.

A FACS experiment, using intracellular staining, was performed with Lad-2 cells incubated with mouse anti-4-1BB mAb or mouse isotype control IgG mAb, either in medium with 10% FBS conditioned with SK-MEL-30 cells or 10% FBS, medium as negative control. Serum free medium or serum free medium with calcium ionophore were also used as controls. Calcium ionophore was added as a positive control to trigger 4-1BB gene expression by increasing the intracellular Ca^{2+} levels. After twenty-four hours incubation a shift in Lad-2 cells incubated with mouse anti-4-1BB mAb in serum free medium with calcium ionophore was seen. There were also higher amount of isotype control antibody bound to the Lad-2 cells grown in serum free medium. No other differences between Kat-4 conditioned or control mediums or the 4-1BB and the isotype control antibody were seen. Figure 5 shows expression of 4-1BB and IgG after twenty-four hours in Lad-2 cells either incubated in medium with 10 % FBS, medium with 10% FBS conditioned with SK-MEL-30 cells, serum free medium or serum free medium with calcium ionophore.

To investigate the gene expression of 4-1BB in human mast cells, real time-PCR was used. In the first experiment HMC-1 cells were either incubated in medium conditioned with Kat-4 or SK-MEL-30 cells. 4-1BB was up-regulated in HMC-1 cells incubated for four hours in medium conditioned with Kat-4 cells but not in HMC-1 cells incubated in medium conditioned with SK-MEL-30 cells. No difference was seen between HMC-1 cells incubated in control medium (Figure 6A). This indicates that Kat-4 cells can stimulate mast cells to express 4-1BB.

Since this result was provided first after collecting cells for the FACS experiments, SK-MEL-30 cells were chosen as medium stimulants for the next Real time-PCR experiment. In the next experiment Lad-2 cells were used since they are more alike peripheral mast cells than HMC-1 cells are. Calcium ionophore was used as positive control to establish that the mast cells can express 4-1BB.

In the second Real time-PCR experiment, Lad-2 cells were either incubated in medium conditioned with SK-MEL-30 or its corresponding control medium, serum free medium or serum free medium with calcium ionophore. 4-1BB was up-regulated in Lad-2 cells incubated for four hours in medium with 10% FBS and in medium with 10% FBS conditioned with SK-MEL-30 cells. This could indicate a serum effect on Lad-2 cells, which are usually cultured in serum free medium.

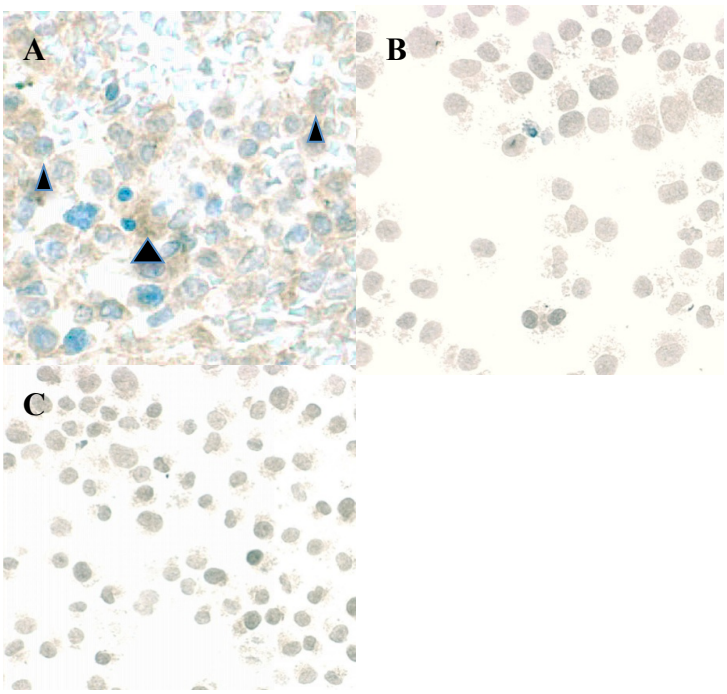

Figure 4A. Immunohistology. Human spleen incubated with rabbit anti-4-1BB mAb. Positive cells, black arrows, with brown cytoplasma. B. HMC-1 cells incubated for zero hours in medium conditioned with Kat-4 cells, cytospin specimen incubated with rabbit anti-4-1BB mAb. No distinctly positive cells. C. HMC-1 cells incubated for four hours in medium conditioned with Kat-4 cells, cytospin specimen incubated with rabbit anti-4-1BB mAb. No distinctly positive cells.

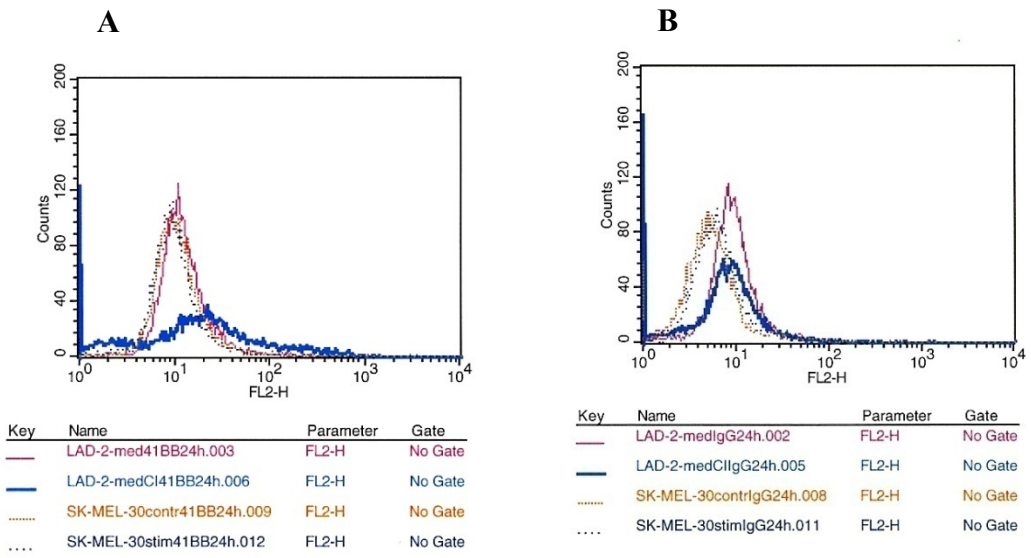

Figure 5. FACS, Intracellular staining of Lad-2 cells. A. Expression of 4-1BB in cells incubated for twenty-four hours in serum free medium (pink), medium with calcium ionophore (clear blue), medium with 10% FBS (yellow) and medium with 10% FBS conditioned with Kat-4 cells (dark blue). A shift in Lad-2 cells incubated in serum free medium with calcium ionophore was seen (clear blue). B. Isotype control antibody binding to cells incubated for twenty-four hours in serum free medium (pink), serum free medium with calcium ionophore (clear blue), medium with 10% FBS (yellow) and medium with 10% FBS conditioned with Kat-4 cells (dark blue). No significant differences in expression of IgG were seen between mediums.

Lad-2 cells incubated in serum free medium with calcium ionophore up-regulated their gene expression of 4-1BB after twenty-four hours, which means human mast cells most likely express 4-1BB and calcium ionophore works as a positive control (Figure 6B). Since Lad-2 cells stimulated with SK-MEL-30 do not express 4-1BB in this experiment, Lad-2 cells stimulated with Kat-4 cells were used in the next experiment. Lad-2 cells are normally cultured in serum free medium and in the next experiment only serum free medium were used. At time zero, no gene up-regulation should be seen and, because of that, only Lad-2 cells incubated in serum free medium were used at that time point in the next experiment.

In the third Real time-PCR experiment, Lad-2 cells were either incubated in serum free medium, serum free medium conditioned with Kat-4 cells or serum free medium with calcium ionophore. At time zero, no up-regulation at the mRNA level was seen. Calcium ionophore served as a positive control. A t-test showed a statistically significant difference between Lad-2 cells incubated for four hours in serum free medium and Lad-2 cells incubated for four hours in serum free medium conditioned with Kat-4 cells. In this experiment biological duplicates were used (Figure 6C). This experiment shows that human mast cells have an up-regulated gene expression of 4-1BB when they are exposed to medium conditioned with Kat-4 cells. A significant up-regulation of 4-1BB is seen after four hours. After twenty-four hours the gene expression has decreased, in agreement with the results from a study by Nishimoto *et al.* in 2005.

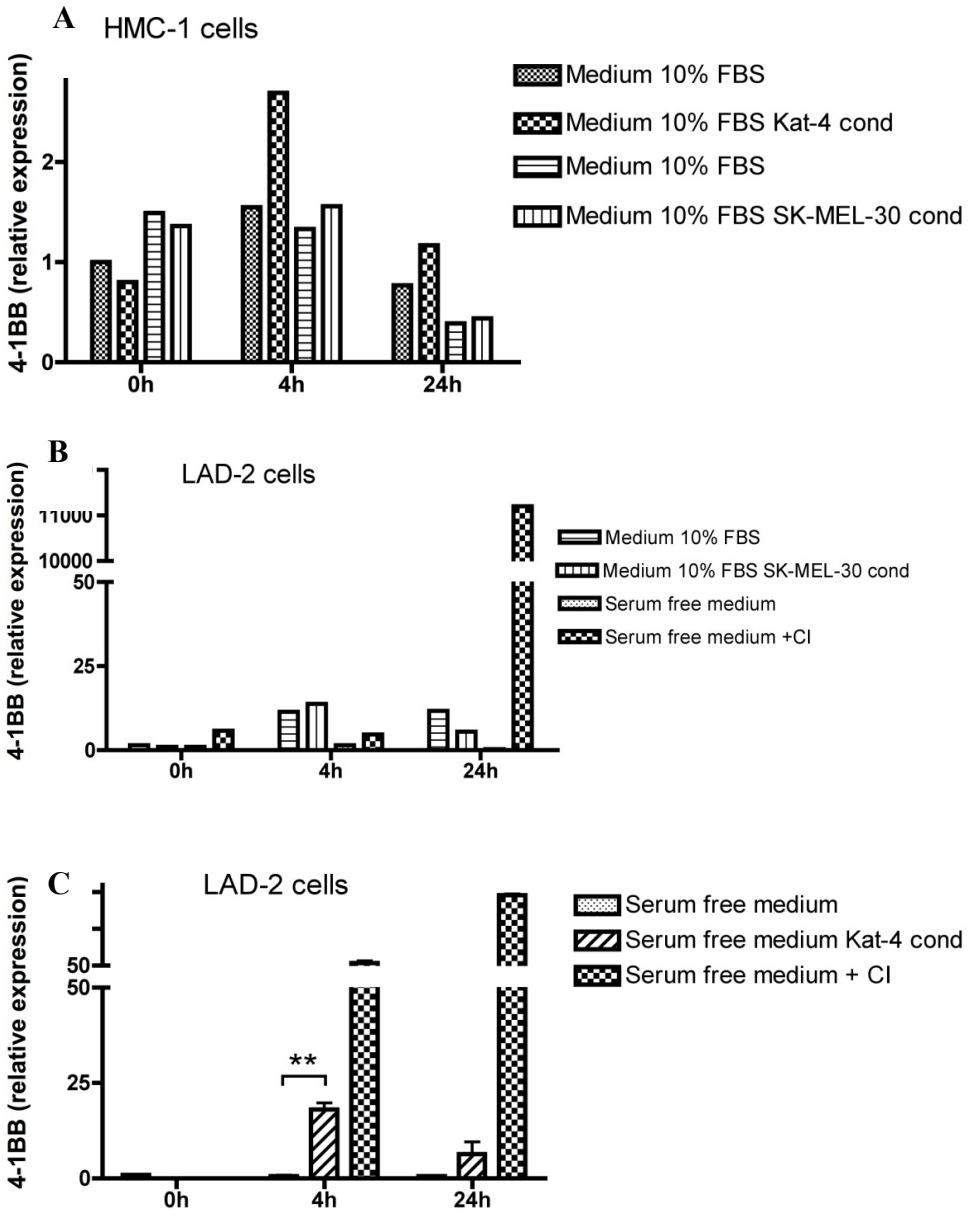

Figure 6. A. An up-regulation of 4-1BB in HMC-1 cells incubated in medium with 10% FBS is seen. The reason for using medium with 10% FBS two times in the experiment is that the cells were collected at two different time points and each medium with 10% FBS is a control to respective conditioned medium. B. The up-regulation of 4-1BB in Lad-2 cells incubated in medium with 10% FBS and in medium with 10% FBS conditioned with SK-MEL-30 cells is probably a serum effect since Lad-2 cells usually are cultured in serum free medium. Calcium ionophore serves as positive control. C. A significant difference in expression of 4-1BB is found between Lad-2 cells incubated with mouse anti-4-1BB mAb for four hours in serum free medium and in serum free medium conditioned with Kat-4 cells. Serum free medium serves as a negative control and serum free medium with calcium ionophore serves as a positive control.

An immunohistology experiment was carried out with Lad-2 cells either incubated in serum free medium, serum free medium conditioned with Kat-4 cells or serum free medium with calcium ionophore. Human spleen served as a positive control. The antibodies used were: rabbit anti-4-1BB pAb and rabbit IgG pAb (served as a negative control). TBS-T (no primary antibody) served as a negative control. In human spleen few 4-1BB positive cells were found, fewer than in earlier specimens, which could indicate preparation problems. When comparing cytospin specimens no significant differences in 4-1BB protein expression were seen between Lad-2 cells incubated for zero hours in serum free medium and Lad-2 cells incubated for four respective twenty-four hours in serum free medium conditioned with Kat-4 cells. Lad-2 cells incubated for twenty-four hours in serum free medium with calcium ionophore had more positive cells than Lad-2 cells incubated for zero hours in serum free medium (Figure 7). Some moderately positive cells, i. e. cells expressing 4-1BB, were found in Lad-2 cells incubated for twenty-four hours in serum free medium with calcium ionophore. This indicates that mast cells can express the protein, which also was shown by Nishimoto *et al.* in 2005. In this study no expression of 4-1BB protein is seen in HMC-1 cells or Lad-2 cells incubated in tumor conditioned medium.

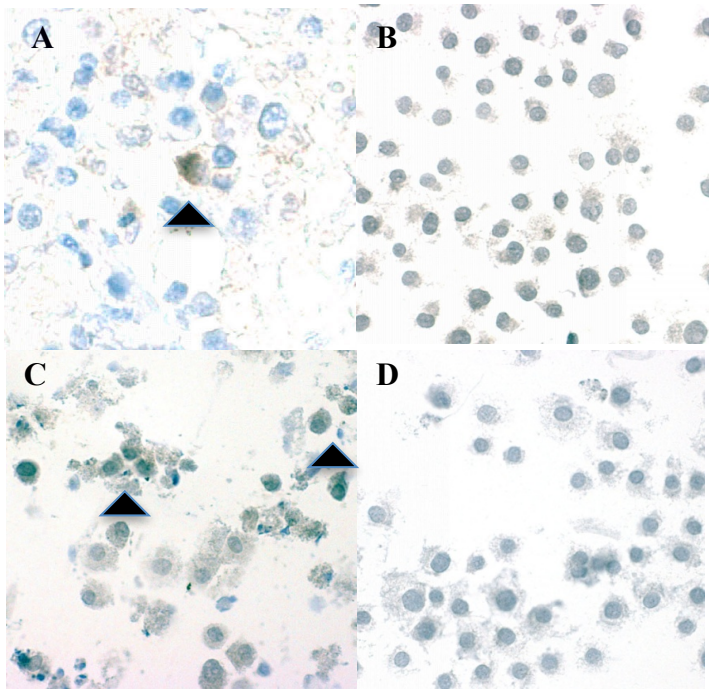

Figure 7. A. Human spleen incubated with rabbit anti-4-1BB mAb. Few positive cells, black arrow. B. Lad-2 cells incubated for zero hours in serum free medium conditioned with Kat-4 cells, cytospin specimen incubated with rabbit anti-4-1BB mAb. No distinctly positive cells are found. C. Lad-2 cells incubated for twenty-four hours in serum free medium with calcium ionophore, cytospin specimen incubated with rabbit anti-4-1BB mAb. Some moderately positive cells, black arrows, are found. D. Lad-2 cells incubated for twenty-four hours in serum free medium conditioned with Kat-4 cells, cytospin specimen incubated with rabbit anti-4-1BB mAb. No distinctly positive cells are found.

DISCUSSION

In the first FACS experiment (Figure 3), performed with cell surface staining, no expression of 4-1BB was detected in HMC-1 cells incubated with tumor conditioned medium. By using this method even a low protein expression should be found. If there is no technical problem or problem with the preparations it is reasonable to assume that human mast cells do not express 4-1BB on their cell surface. If they express the protein intracellularly that can be discovered by using an intracellular staining in the next FACS experiment. The next FACS experiment was therefore performed with intracellular staining. In that experiment Lad-2 cells was used instead of HMC-1 cells since they are more like peripheral mast cells.

In the first immunohistology experiment (Figure 4), no expression of 4-1BB was seen in HMC-1 cells incubated in medium conditioned with Kat-4 cells. A study showed that 4-1BB is expressed on the cell surface of T cells (Yunle *et al*., 2003). In human spleen 4-1BB was expressed in some cells and the rabbit anti-4-1BB pAb was shown to work. There were cells with a high expression of 4-1BB, cells with a quiet low expression of 4-1BB and cells with no expression of 4-1BB. The rabbit Ig pAb did not work, which made it hard to find the specific expression of 4-1BB. There can be several reasons for these problems, for example with the antibodies, the preparations and with the HMC-1 cells. Therefore, the next immunohistology experiment was carried with Lad-2 cells, since they are more like peripheral mast cells.

In the second FACS experiment (Figure 5), carried out with intracellular staining, no expression of 4-1BB was detected in Lad-2 cells incubated in medium conditioned with SK-MEL-30. Since neither HMC-1 cells nor Lad-2 cells showed an expression of 4-1BB mRNA it was not reasonable to expect a protein expression of 4-1BB. The shift in expression of 4-1BB in Lad-2 cells incubated for twenty-four hours in serum free medium with calcium ionophore indicates that mast cells can be induced to express the protein, which also was shown by Nishimoto *et al.* in 2005. The Fc-block, used to block unspecific binding, was manufactured for use on mouse cells and not human cells. Probably it does not work on human cells. To optimize this method, the Fc-block should be changed into one that is manufactured for use on human cells. It would then be interesting to perform a FACS experiment, carried out with cell surface staining, using Lad-2 cells incubated in medium conditioned with Kat-4 cells. If that does not work, an experiment with intracellular staining of Lad-2 cells conditioned with Kat-4 cells can be performed.

In the first Real time-PCR experiment (Figure 6A), an up-regulation of 4-1BB was seen after four hours in HMC-1 cells incubated in medium conditioned with Kat-4 cells. This indicates that human mast cells can express 4-1BB. In the second Real time-PCR experiment (Figure 6 B), there seemed to be a serum effect on Lad-2 cells expressing 4-1BB both when they were stimulated with or without medium conditioned with SK-MEL-30 cells.

The use of a positive control, Lad-2 cells incubated in serum free medium with calcium ionophore, showed that human mast cells can induce the expression of 4-1BB. These two results together led to the third real time-PCR experiment (Figure 6C), where Lad-2 cells incubated in serum free medium conditioned with Kat-4 cells showed a significant up-regulation of 4-1BB after four hours. After twenty-four hours, gene expression decreased. A study showed that mast cells sensitized with IgE over night have an up-regulated gene expression of 4-1BB (Nishimoto *et al*., 2005). The same study showed that mast cell protein expression of 4-1BB reaches its highest levels after 8 hours sensitization with IgE and after 12 hours the expression is reduced. Further, it was shown that the level of 4-1BB decreased during the incubation time if no more stimulation was done. That result is similar to this study's result, where Lad-2 cells incubated in serum free medium conditioned with Kat-4 cells up-regulate 4-1BB after four hours and start to reduce the expression within twenty-four hours. Mast cells incubated with calcium ionophore have an increased gene expression after twenty-four hours compared to the expression after four hours. In this study I show that neither HMC-1 cells nor Lad-2 cells incubated with SK-MEL-30 conditioned medium up-regulate their gene expression of 4-1BB. That does not mean an up-regulation of 4-1BB is impossible to find in other cell lines. It is interesting that mast cells accumulate around melanomas (Guidolin *et al.*, 2005) especially since anti-4-1BB mAb is in stage II clinical trials for treatment of melanomas (U.S. National institutes of health. [online], 2010). Guidolin *et. al., 2005* showed that mast cells may be of importance for tumor angiogenesis and thereby promote tumor development. If mast cells have the ability to inhibit some stage of tumor development is still an open question.

In the second immunohistology experiment (Figure 7), no expression of 4-1BB protein was seen in Lad-2 cells incubated in serum free medium conditioned with Kat-4 cells. In Lad-2 cells incubated for twenty-four hours in serum free medium with calcium ionophore, some cells had a moderate expression of 4-1BB. This indicates that mast cells can express the protein, which also was shown by Nishimoto *et al.* in 2005. In this study, no protein expression of 4-1BB has been registered, neither in HMC-1 cells nor in Lad-2 cells incubated in medium conditioned with Kat-4 cells. A reason for this can be that the rabbit anti-4-1BB pAb does not work properly, e.g if it is too unspecific. Another cause can be problems with the fixation of the cytospin specimens. Since the gene expression of 4-1BB is quite low in Lad-2 cells incubated in serum medium conditioned with Kat-4 cells, the protein expression can also be low. This method probably needs a certain rate of expression to find to the protein and, if that rate is to low, that can be the reason for not observing any expression of 4-1BB. Lad-2 cells incubated in serum medium with calcium ionophore, which has a much higher rate of gene expression of 4-1BB, shows some moderately positive cells. The cytospin specimens with mast cells incubated with rabbit IgG pAb are overexposed since the antibody does not work properly. A probable reason was insufficient dilution of the antibody, which can cause high unspecific binding of the antibody. This antibody was not prepared by the same manufacturer or in the same way as the rabbit anti-4-1BB pAb. This can be the reason why it does not work as expected.

The rabbit anti-4-1BB pAb seems to work, it works in the positive control, and therefore the reason for it not working in the cytospin specimens should be determined. The fixation procedure and the rate of expression of 4-1BB differ between the positive control and the cytospin specimens. Since the rate of expression cannot be manipulated, the fixation procedure for the cytospin specimens may be of particular interest.

My results show that in the human mast cell models I used it was possible to detect increased mRNA levels of 4-1BB when the mast cells where incubated in tumor conditioned medium. However, it was not possible to prove a connection between gene expression and increased expression of 4-1BB protein in mast cells exposed to tumor factors. If there are no technical reasons for the difference it is an additional example of the known fact that mRNA and protein expression is not always directly correlated. More studies are required to resolve if my results are a general phenomenon or if they are dependent on the cell models.

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REFERENCES

Carlini, M. J., Dalurzo, M. C. L., Lastiri, J. M., Smith, D. E., Vasallo, B. C., Puricelli, L. I. and de Cidre L. S. L. (2010). Mast cell phenotypes and microvessels in non–small cell lung cancer and its prognostic significance. Human Pathology 41, 697–705.

Coussens, L. M., Raymond, W. W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., Caughey, G. H. and Hanahan, D. (1999). *Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis*. Genes and development 13, 1382–1397.

Grewal, I. S. (Ed.). (2009). *Therapeutic Targets of the TNF Superfamily*. New York: Springer Science and Business Media, LLC Landes Bioscience

Guidolin, D., Crivellato, E., Nico, B., Andreis, P. G., Nussdorfer G. G. and Ribatti D. (2006). *An image analysis of the spatial distribution of perivascular mast cells in human melanoma*. International journal of molecular medicine 17, 981-987.

Guhl, S., Babina, M., Neou, A., Zuberbier, T. and Artuc, M. (2010). Experimental Dermatology 19, 845–847. *Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells – drastically reduced levels of tryptase and chymase in mast cell lines.*

Husmark, J., Heldin, N.E. and Nilsson, M. (2002). *Follicular growth of a thyroid carcinoma cell line (KAT-4) with abnormal E-cadherin and impaired epithelial barrier.* Thyroid 12 (9), 781-790.

Kwon, B. S. and Weissman, S. M. (1989). *cDNA sequences of two inducible T-cell genes*. Proc. Natl. Acad. Sci. 86, 1963-1967.

Lu, X., Jiang, X., Liu, R., Zhao, H. and Liang, Z. (2008). *Adoptive transfer of pTRP2-specific CTLs expanding by bead-based artificial antigen-presenting cells mediates anti-melanoma response*. Cancer Letters 271, 129–139.

Lu, X-L., Jiang, X-B., Liu, R-E. and Zhang, S-M. (2009). *In vivo anti-melanoma eYcacy of allo-restricted CTLs specific for melanoma expanded by artificial antigen-presenting cells*. Cancer Immunol Immunother 58, 629–638.

Maltby, S., Khazaie, K. and McNagny, K. M. (2009). *Mast cells in tumor growth: Angiogenesis, tissue remodelling and immune-modulation*. Biochimica et Biophysica Acta 1796, 19–26.

Miller, R. E., Jones, J., Le, T., Whitmore, J., Boiani, N., Gliniak, B. and Lynch D. H. (2002). 4-1BB-Specific Monoclonal Antibody Promotes the Generation of Tumor-Specific Immune Responses by Direct Activation of CD8 T Cells in a CD40-Dependent Manner. J Immunol 169, 1792-1800.

Molecular station. Home page. Available from: http://www.molecularstation.com/real-time-pcr/#realtimeintro, 2010-11-25

Murillo, O., Arina, A., Hervas-Stubbs, S., Gupta, A., McCluskey, B., Dubrot, J., Palazón, A., Azpilikueta, A., Ochoa, M. C., Alfaro, C., Solano, S., Pérez-Gracia, J. L.,Oyajobi, B. O. and Melero, I. (2008). *Therapeutic anti-tumor efficacy of anti-CD137 agonistic monoclonal antibody in mouse models of myeloma*. Clin Cancer Res. Author manuscript; available in PMC 2009 November 1.

Nishimoto, H., Lee, S-W., Hong, H., Potter, K. G., Maeda-Yamamoto, M., Kinoshita, T., Kawakami, Y., Mittler, R. S., Kwon, B. S., Ware, C. F., Croft, M. and Kawakami, T. (2005) *Costimulation of mast cells by 4-1BB, a member of the tumor necrosis factor receptor superfamily, with the high-affinity IgE receptor*. Blood J. 106(13), 4twenty-four1-4twenty-four8.

Oldford, S. A., Haidl, I. D., Howatt, M. A., Leiva, C. A., Johnston, B. and Marshall, J. S. (2010). *A Critical Role for Mast Cells and Mast Cell-Derived IL-6 in TLR2-Mediated Inhibition of Tumor Growth*. J. Immunol. 185, 7067-7076.

Ribatti, D., Guidolin, D., Marzullo, A., Nico, B., Annese, T., Benagiano, V. and Crivellato, E. (2010). *Mast cells and angiogenesis in gastric carcinoma*. Int. J. Exp. Path.

Romano, P., Manniello, A., Aresu, O., Armento, M., Cesaro, M., Parodi, B (2009). *Cell Line Data Base: structure and recent improvements towards molecular authentication of human cell lines.* Nucleic Acids Research 37(Database issue):D925-D932. DOI: doi:10.1093/nar/gkn730; PMID: 18927105

Sawatsubashi, M., Yamada, T., Fukushima, N., Mizokami, H., Tokunaga, O. and Shin, T. (2000). *Association of vascular endothelial growth factor and mast cells with angiogenesis in laryngeal squamous cell carcinoma*. Virchows Arch. 436, twenty-four3–twenty-four8.

Schwarz, H. (2005). Biological activities of reverse signal transduction throughCD137 ligand. J. Leukoc. Biol. 77, 281-286.

Soucek, L., Lawlor, E. R., Soto, D., Shchors, K., Brown Swigart, L. and Evan G. I. (2007). *Mast cells are required for angiogenesis and macroscopic* expansion of Myc-induced pancreatic islet tumors. Nature medicine 13 (10), 1211-1218.

U.S. National institutes of health. [online] (2010-12-02). Available from: http://clinicaltrials.gov/ct2/show/study/NCT00612664?show_locs=Y#locn [2011-01-02]

Xiang, M., Gu, Y., Zhao, F., Lu, H., Chen, S. and Yin, L. (2010). *Mast cell tryptase promotes breast cancer migration and invasion*. Oncol. reports 23, 615-619.

Yoshii, M., Jikuhara, A., Mori, S., Iwagaki, H., Takahashi, H. K., Nishibori, M. and Tanaka N. (2005). *Mast Cell Tryptase Stimulates DLD-1 Carcinoma Through Prostaglandinand MAP Kinase-Dependent Manners*. J Pharmacol Sci 98, 450 – 458.

Yunle, W., Shusen, Z., Changku, J., Jiayin, Y., Xiaoling, J. and Zhicheng, Z. (2003). *4-1BB gene expression in peripheral blood mononuclear cells from orthotopic liver transplant recipients with graft acceptance.* Chinese Medical Journal 116 (12), 1854-1859.