Introduction

Multiple Myeloma (MM) is a hematopoietic disease characterized by the expansion of neoplastic plasma cells and is one of the most commonly diagnosed hematological tumors worldwide. Clinical manifestations of the malignancy involve renal impairment, anemia, hypercalcemia and bone destruction. Despite the fact that treatment strategies have drastically improved during the last few years, MM is still considered incurable. Several genetic abnormalities have been associated with the development of MM, including point mutations, translocations and aberrations of whole chromosomes. The inhibitory adaptor protein Src homology domain 3 lymphocyte protein 2 (SLy2) is located on chromosome 21q11.2 and belongs into a group of genes frequently disrupted in MM patients. However, the role of SLy2 in intracellular plasma cell signaling and its involvement in MM development still remain unclear. In this study, we examine the expression profile of SLy2 in different human MM cell lines and discuss its possible contribution to Histone Deacetylase (HDAC)-mediated gene repression in MM.

Keywords: HDAC; SLy2; Multiple myeloma; MGUS

Abstract

Multiple Myeloma (MM) is a heterogeneous disease of the hematopoietic system that is characterized by the expansion of neoplastic plasma cells and is one of the most commonly diagnosed hematological tumors worldwide. Clinical manifestations of the malignancy involve renal impairment, anemia, hypercalcemia and bone destruction. Despite the fact that treatment strategies have drastically improved during the last few years, MM is still considered incurable. Several genetic abnormalities have been associated with the development of MM, including point mutations, translocations and aberrations of whole chromosomes. The inhibitory adaptor protein Src homology domain 3 lymphocyte protein 2 (SLy2) is located on chromosome 21q11.2 and belongs into a group of genes frequently disrupted in MM patients. However, the role of SLy2 in intracellular plasma cell signaling and its involvement in MM development still remain unclear. In this study, we examine the expression profile of SLy2 in different human MM cell lines and discuss its possible contribution to Histone Deacetylase (HDAC)-mediated gene repression in MM.

Keywords: HDAC; SLy2; Multiple myeloma; MGUS

Introduction

Multiple Myeloma (MM) is a hematopoietic disease characterized by malignant growth of clonal plasma cells, infiltrating the bone marrow [1]. It is a low-grade B-cell lymphoma that accounts for about 10% of all hematopoietic tumor malignancies. MM is a disease of the older ages and is almost always proceeded by a pre-malignant state that is termed Monoclonal Gamopathy of Undetermined Significance (MGUS) [1].

MGUS is defined as a physiological state characterized by <10% of monoclonal plasma cells in the bone marrow, <30 g/l serum monoclonal immunoglobulin protein and by the absence of end organ damage. In approximately 3% of all people with >50 years of age, MGUS is present. Between MGUS and MM there is a state called smoldering myeloma, which is defined as MM without end organ damage [2,3].

Clinical manifestations in MM itself are highly heterogeneous with a strong variation in severity of symptoms, exacerbating diagnostic routines [4]. Symptoms can include loss of weight, hypercalcemia, renal dysfunction, osteolytic bone lesions and bone pain. MM is diagnosed if following conditions are complied: The proportion of monoclonal plasma cells accounts for ≥ 10% of bone marrow cells, monoclonal protein is detectable in the serum or the urine and end-organ damage is in place. End-organ damage is defined by the so called CRAB-criteria (hypercalcemia, renal failure, anemia and lytic bone lesions) [5,3].

With regard to treatment of MM patients, an obvious improvement in medication became apparent over the last few years, especially when it comes to immune-modifying drugs and proteasome inhibitors such as Thalidomide and Bortezomib, respectively. However, despite the increase in availability and effectiveness of new therapeutic drugs, MM is still considered to be in incurable [6].

There are several genetic abnormalities that have been associated with the malignant growth of plasma cells in MM patients. These involve the gain and loss of whole chromosomes, chromosomal...
SLy2 is an inhibitor of B-cell immunity and is significantly up regulated in B cells upon their activation in vitro [11,12]. In 2014, our group reported that SLy2 functions as an important inhibitor of natural B-1 cells [13]. In SLy2-overexpressing mice, the number of B-1 cells is strongly decreased and the response towards pneumococcal vaccine is dampened. As SLy2 is significantly over expressed in Down’s Syndrome patients (DS) due to an additional amplification in the genome, it is probably leading to enhanced susceptibility of DS patients towards pneumococcal infection [13,14]. Complementary, it could also be shown that SLy2-knockout mice display enhanced numbers of B-1 cells as well as increased responses towards vaccination with T-cell independent antigens [15].

However, still little is known about the molecular interaction partners of SLy2 and the signaling cascades it is involved in. In 2010, Brandt and colleagues found that SLy2 is directly targeted by partners of SLy2 and the signaling cascades it is involved in. In a Malaysian study in 2012, several genes have been identified to be located within chromosomal aberration regions in 105 myeloma patients. One of those genes has been shown to be the adaptor protein Src homology domain 3 lymphocyte protein 2 (SLy2), also termed SAMS N1 or HACS1 [8]. SLy2 belongs into a group of three highly homologous adaptor proteins that are involved in several lymphocyte regulatory pathways. It is encoded on Chromosome 21q11.2, a chromosomal region frequently disrupted in hematological diseases. The SLy2-protein is mainly expressed in lymphocytes, but can also be found at lower levels in heart, brain, muscle tissue and endothelial cells. It can be detected both in the cytoplasm as well as inside the nucleus. Therefore, the interaction of SLy2 with 14-3-3 proteins is likely to regulate its sub cellular location [11]. Additionally, SLy2 has been shown to be involved in the regulation of actin dynamics and B-cell spreading through direct interaction with the actin-binding protein cortactin [17].

Once entered the nucleus, SLy2 was found to associate with members of the Sin3-protein complex, including Sin3A-Associated Protein (SAP30) and Histone Deacetylase 1 (HDAC1) [11]. HDACs are enzymes that catalyze the removal of acetyl groups from N-terminal histone residues, thereby stabilizing the chromatin structure and repressing gene transcription. HDACs can directly exert their enzymatic function or can be indirectly involved as a part of molecular signaling complexes [18]. One crucial function of HDACs is the suppression of the anti-proliferative factors p53 and p27, thereby promoting cell cycle progression and preventing apoptotic events [19]. HDAC-inhibitors are widely used as anticancer drugs, with promising effects also in MM patients [20]. Recently, regulation of gene transcription by class I HDACs and HDAC6 has been brought into context with MM, as it has been shown that they regulate the expression of surface-CD38 on MM cells, a marker that correlates with clinical outcome in patients [21]. Interestingly, when co-transfected into HEK293T-cells, SLy2 significantly increased the enzymatic activity of HDAC1, suggesting a putative role of SLy2 in HDAC-mediated gene regulation [11].
Intriguingly, Amend and colleagues could report a large chromosome deletion in MM patient samples affecting the whole SLy2-coding gene section and probably leading to a total knockout of SLy2 [22]. Furthermore, Noll et al. reported a significant reduction of SLy2-expression in C57BL/KaLwRij mice, a strain that is known to spontaneously develop MM-like disease. The group could also report a significant down regulation of the SLy2-protein in CD138+ plasma cells derived from MM patients if compared to MGUS subjects [23].

Taken together, these data strongly suggest a role of SLy2 as a tumor suppressor gene, possibly down regulated during MM development. Therefore, SLy2 constitutes a potential therapeutic future target for drug development in molecular therapy of B-cell disorders.

Materials and Methods

Cell culture, stimulation and transfection

Human JK6L, U266 and RPMI 8226 cells were cultured in HyClone RPMI-1640 medium (GE Healthcare, München) with supplements (10% FCS, 1% Penicillin/Streptomycin, 2 mM L-Glutamin) and incubated at 37°C and 5% CO₂. Cell line passage was performed at least twice a week, depending on the density of cells. For stimulation, cells were seeded into 6-well plates and 10 ng/mL APRIL and/or IL-6 (Bio-techne, Wiesbaden-Nordenstadt) was added. In vitro stimulation took place for 24 hrs.

Transient transfection of cells was done using the RPMI 8226 Cell Acalanche Transfection Reagent (EZ Biosystems, Maryland). For this purpose, 40 pmol of corresponding siRNA (SAMSNI Silencer® select pre-designed siRNA or Silencer® select negative control #1 siRNA, by Thermo Fisher Scientific, Waltham) and 2 μl to 3 μl of the transfection reagent were applied onto a cell layer consisting of 5 × 10⁵ cells. Upon treatment, plates were briefly centrifuged and incubation took place for 24 hrs at 37°C and 5% CO₂. Success of transfection was verified using RT-PCR.

Protein lysates

Cell lysates for biochemistry were prepared from cultured cells after 24 hrs of stimulation or transfection. Per approach, 150 μL of lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% NP-40 (v/v)) freshly supplemented with phosphatase and protease inhibitor (Roche, Mannheim) was used. Lysis was performed on ice for 60 mins. Subsequently, samples were centrifuged and supernatants containing protein contents were collected. Protein concentration was determined using the DC™ protein assay (Bio-Rad, Puchheim).

For performance of western blot, 25 μg protein/sample were loaded onto the gel and nitrocellulose membranes were prepared by wet blotting. Chemiluminescent imaging was done using the SignalFire™ Elite ECL Reagent (Cell Signaling, Frankfurt am Main) and the VersaDoc was applied for analysis.

RT-PCR

Isolation of total RNA from stimulated or transfected cell culture was performed according to the NucleoSpin® RNA-Kit (Macherey-Nagel, Düren). Subsequently, RNA-concentration was measured with the NanoDrop and cDNA-transcription was done using the High capacity cDNA reverse transcription kit (Applied Biosystems, Waltham). RT-PCR was performed with the Roche LightCycler® 480 using the SensiFAST SYBR No-ROX Mix (Bioline, London). Specific primers (Biomers, Ulm) targeting β-actin (5’-ccgacaggatgcagaagg-3’ and 5’-aggaaagacacccaccttga-3’), p21 (5’-ccgaagtcagttccttgtgg-3’ and 5’-catgggttctgacggacat-3’), p27 (5’-tttgacttgcatgaagagaagc-3’ and 5’-agctgtctctgaaagggacatt-3’), p53 (5’-agtctagagccaccgtccag-3’ and 5’-agtgacccggaaggcagt-3’), p57 (5’-ctcctttccccttcttctcg-3’ and 5’-tccatcgtggatgtgctg-3’), STAT3 (5’-cccttggattgagagtcagag-3’ and 5’-aagcggctatactgctggtc-3’) and SLy2 (5’-cctcttgtattcgccacaca-3’ and 5’-cgaaattcccaaaactgctg-3’) were used.

HDAC-activity assay

Protein lysates were prepared as described above. To measure
the overall HDAC-activity in the samples, the HDAC Assay Kit (Millipore, Darmstadt) was applied according to the manufacturer’s protocol provided. Subsequently, the fluorescent output was measured at an excitation wavelength of 390 nm and an emission wavelength of 460 nm using the victor 2.

Statistical analysis
Statistical analysis was performed as indicated in each of the corresponding figure legends. In all cases, a p-value of <0.05 was considered statistically significant. Data are shown as mean ± SD.

Results and Discussion
SLy2-Expression is significantly up regulated in RPMI 8226 and JK6L cells upon stimulation in vitro

In this study we have examined the impact of in vitro stimulation of MM cell lines on their intrinsic expression of the adaptor protein Sly2. To this end, U266, JK6L and RPMI 8226 cells were stimulated using IL-6 and APRIL, which are essential stimulators and survival factors during plasma cell development [24]. Cells were either stimulated separately with IL-6 or APRIL, or with a combination of both. Subsequently, Sly2-expression levels were determined by quantitative RT-PCR. As shown in Figure 1A, expression levels of Sly2 slightly increased in U266 cells upon stimulation, but no significant changes were induced. By contrast, our experiments reveal a significant induction of Sly2-expression in JK6L and RPMI 8226 cells upon stimulation with a combination of IL-6 and APRIL (Figure 1B and 1C). In RPMI 8226 cells, APRIL alone was not sufficient to elicit significant changes in expression, whereas activation using IL-6 induced a significant upregulation of Sly2 (Figure 1C). These results are consistent with previous findings, demonstrating that Sly2 is induced upon in vitro stimulation of B-cells [12] and further demonstrate, that this effect can also be found in human MM cell lines under certain conditions. As the alterations in Sly2-expression upon activation using IL-6/APRIL were most strongly marked in RPMI 8226 cells, we decided to subsequently focus our experiments on this cell line.

IL-6/APRIL-mediated induction of Sly2 in RPMI 8226 cells is accompanied by a regression in overall HDAC-activity

In 2010, our group has demonstrated that co-expression of Sly2 in transfected HEK293T cells increases the activity of HDAC1 [11]. As already mentioned above, HDACs serve as important regulators of epigenetic gene modification. By remodeling the chromatin structure or being part of huge signaling complexes, they are able to efficiently modulate gene transcription [25,26]. Hence, we wanted to investigate whether Sly2-induction in activated RPMI 8226 cells may also be accompanied by alterations in HDAC-activity and set out to examine overall HDAC-activity levels. Surprisingly, stimulation with IL-6 and APRIL or APRIL alone led to a reduction of overall HDAC-activity in RPMI 8226 cells (Figure 2). Of importance, while specifically testing HDAC1-activity in terms of the first study, we here observed the overall activity of several HDAC-family members at once. As it has been reported that there exist significant differences of the function of related HDACs, a direct comparison of both studies is not possible [27]. For instance, a homozygous knockout of HDAC1 in mice leads to embryonic lethality, despite the fact that HDAC2 and 3 are unregulated upon the knockout [25]. This strongly indicates that the loss of HDAC1-specific signaling functions cannot be fully compensated by other members of the HDAC-family, and therefore proves that they harbor distinct functional capacities. We therefore plan to include examination of the specific activity of HDAC1 in our future experiments. Moreover, a cell type- and tissue-specific phenotype of HDAC-activity is very likely.

In literature, there already exist some studies specifically focusing on the function of HDACs in human MM cell lines. For example, treatment of MM-1S cells using the broad HDAC-inhibitor Suberoylanilide Hydroxamic Acid (SAHA) was reported to suppress proliferation and survival in these cells, accompanied by an upregulation of pro-apoptotic factors such as p53 [28]. This suggests an anti-apoptotic and pro-proliferative function of HDACs in human MM cells. Thus, we next wanted to elucidate further effects of the HDAC-downregulation on the expression of several important HDAC-target genes in stimulated RPMI 8226 cells.
IL-6/APRIL-dependent stimulation of RPMI 8226 cells induces alterations in HDAC-target gene expression

HDACs are known to influence lots of distinct genes that are mainly involved in cell cycle regulation and apoptosis induction [18]. Therefore, we examined the expression levels of different HDAC-target genes in stimulated RPMI 8226 cells, including p57, p21, p27, p53 and STAT3. As depicted in Figure 3A and 3B, no significant changes in expression of p21 and p53 were observed upon stimulation of RPMI 8226 cells.

By contrast, we did observe significant changes in expression of p27 upon in vitro activation (Figure 3C). p27 is an important HDAC-target that belongs into the group of Cyclin Kinase Inhibitors (CKI) and negatively regulates cell cycle progression [29].

Concomitant with the reduction in overall HDAC-activity through IL-6/APRIL-mediated stimulation, levels of p27-expression increased in RPMI 8226 cells (Figure 3C). This finding is in consistence with a previous report by Pang and colleagues, who could show that silencing of HDAC2 leads to an increase in p27-protein [19]. Of importance, p27-expression has been shown to only underlie the control of HDAC2 alone [19].

Another HDAC-target of our interest is Signal Transducer and Activator of Transcription 3 (STAT3). Cytoplasmic STAT proteins serve as downstream signal transducer of transmembrane receptors (e.g. the IL-6 receptor) and are phosphorylated upon their activation, mainly Janus Kinase (JAK). In their activated state they form homo- and heterodimers and translocate into the nucleus to regulate gene transcription [30]. Here we found, that simultaneously with SLy2-induction through IL-6-mediated stimulation the expression of STAT3 was significantly enhanced (Figure 3D). Again, this effect could not be achieved by stimulation with APRIL alone. This finding is consistent with previous studies, showing that IL-6 is an important activator of STAT3 in human MM cells [31]. Of interest, STAT3 has been reported to be constitutively active in U226 cells as well as in bone marrow derived from human MM patients [32]. As visible in Figure 3D, STAT3-transcription does not seem to be constitutively active in RPMI 8226 cells, but can be highly induced through the addition of IL-6. However, according to literature, an induction of STAT3-activity likely goes ahead with enhanced HDAC1-activity [19,33]. Of note, our assay only focused on the transcriptional expression of the STAT3-gene and not on the phosphorylation state of STAT3-protein. The transcriptional regulation of STAT3 does not necessarily reflect its activation state in the cytoplasm.

Taken together, future studies should additionally be focused on the activation state of STAT3 in the cytoplasm of IL-6-stimulated RPMI 8226 cells, to figure out a correlation of STAT3-transcriptional regulation and its cytoplasmic phosphorylation state.

Interestingly, along with the increase in STAT3-expression, we observed a significant reduction of p57 under exactly the same conditions (Figure 3E). P57 is a CIK and its accumulation results in cell-cycle arrest [29]. According to literature, HDAC1 and 2 are both able to directly bind to the promoter region of the p57-gene, thereby suppressing its gene expression [34]. To further explain the connection between the increase in p57 and the drop in HDAC-activity that we observe upon stimulation, an overall HDAC-activity assay will not be sufficient. It reflects the activity of more than 10 HDAC-family members at once and may not give indications about the activity of specific HDAC-members.

Induction of STAT3 and downregulation of p57 at the same time strongly suggest an upregulation of HDAC1 rather than a decrease in its activity. The fact that we observe a loss in overall HDAC-activity could overlay changes in specific HDAC1-activity and rather reflect the activity of HDAC2 (which would explain the induction of p27-expression) and other members of the protein family. Still, an upregulation of HDAC1 alone would not be visible within the overall changes of activation levels. The performance of more specific assays...
Overall HDAC-activity and HDAC-target gene expression in SLY2-knockdown cells

To find out whether there is a biological connection between the alterations in HDAC-activity and the upregulation of SLY2, we set out to create SLY2-knockdown cells by transiently transfecting RPMI 8226 cells with small-interfering (si) RNAs. Using SLY2-sequence specific siRNA, we could achieve a significant knockdown of SLY2-expression in siSLY2-transfected cells as compared to control-transfected ones. This was shown on both, transcriptional as well as protein levels (Figure 4A and 4B). Of note, siSLY2-transfection decreased the amount of SLY2 that was expressed, but did not lead to a complete knockout of the protein in these cells.

To further elucidate possible connections between HDAC-activity and SLY2-expression, we performed an overall HDAC-activity assay of the generated SLY2-knockdown cells. Figure 5 illustrates that there is an obvious increase of relative HDAC-activity upon knockdown of SLY2; however this increase was not statistically significant (Figure 5). When looking at the expression of the HDAC-target genes in the SLY2-knockdown cells in the absence of stimulation, we found no significant differences regarding the expression of p21, STAT3 and p57. By contrast, p27-expression levels were still enhanced (Figure 6C) and a significant reduction of p53 was induced (Figure 6B). Of importance, as we could not achieve a complete knockout of SLY2 but only a knockdown, it is very likely that there was still enough SLY2-protein active in the system, exacerbating the assessment of conclusive data. To sum up this part, a complete SLY2-knockout would be necessary to find out more about the role of SLY2 in HDAC-regulation. According to our opinion, assays using SLY2-knockdown cells could give valuable indications about its involvements, whereas a simple knockdown of SLY2 could not clarify our experimental questions in this study.

In summary, our study revealed that SLY2 is significantly unregulated in RPMI 8226 cells stimulated with IL-6, accompanied by a downregulation of overall HDAC-activity that is associated with significant changes in HDAC-target expression on the transcriptional level. To gain further insights into possible connections of SLY2 and the regulation of HDACs in stimulated RPMI 8226 cells, we propose assays specifically focusing on certain members of the HDAC-family, in particular on HDAC1 and 2.

Previous studies revealed that the inhibition of HDAC-activity efficiently induces apoptosis in human MM cells and increases their sensitivity towards common cancer treatments, strongly suggesting that HDAC-deregulation may be involved in disease severity and cancer cell resistance [28]. Therefore, it remains essential to further extend our knowledge about potential inhibitors and regulators of HDAC-activity such as the inhibitory adaptor protein SLY2.

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