Apoptosis and a characteristic marker (CD138) in multiple myeloma

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SUMARRY

The limited success of cancer chemotherapy calls for new and better drugs with more relevance to the regulation of tumor growth and progression. Apoptosis is a critical process in normal B-cell function, and in many cases the abnormalities in the apoptotic pathways contribute to the pathogenesis of B-cell malignancies. This scenario has particular relevance in multiple myeloma (MM).

In the present study we analysed the effect of mevastatin - a novel inhibitor of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway - on U266 human myeloma cells. Apoptosis induced by mevastatin was associated with increased caspase activity and depolarisation of mitochondrial membrane. Expression of BCL-2 mRNA and protein was down-regulated, with no change in BAX or BCL\textsubscript{xL} protein production. The mitochondrial program was supported by caspase-8 and cleaved-BID activity. None of the antibodies neutralizing death-ligand/death-receptor pathway – TRAIL-R2Fc, anti-TNF-\beta, anti FASL (NOK-1) - influenced the mevastatin-induced apoptosis. Mevastatin also stimulated shedding of syndecan-1 from the surface of myeloma cells.

Syndecans are a family of cell surface proteoglycans. In the bone marrow of multiple myeloma patients syndecan-1 is expressed only on the surface of malignant plasma cells. In this study we analyzed the serum level of shed syndecan-1 in three groups of patients with multiple myeloma, solitary plasmacytoma, and monoclonal gammopathy of undetermined significance. Patients with multiple myeloma showed a significantly higher median serum syndecan-1 level than patients with plasmacytoma or monoclonal gammopathy of undetermined significance. Statistically significant differences were also observed among Salmon-Durie subgroups of 50 patients suffering from multiple myeloma. In addition to these findings a statistical correlation with other independent prognostic factors such as serum beta\textsubscript{2} -microglobulin level, monoclonal immunoglobulin concentration, and bone marrow plasma cell count could also be noted. A significant decrease in median serum syndecan level was observed in patients who responded to chemotherapy, whereas no change in the median syndecan-1 level could be observed in nonresponders. Our findings confirm the observation that high serum soluble syndecan-1 level is associated with a more advanced disease stage and is a strong independent indicator of poor prognosis. A diminished serum syndecan-1 reading as a result of chemotherapy may be a good indicator of favorable response to antitumor treatment.
INTRODUCTION

The limited success of cancer chemotherapy calls for new and better drugs with more relevance to the regulation of tumor growth and progression. Generally, antiproliferative and proapoptotic functions are still the leading targets, but DNA has to share the center stage with other, mainly signal transducing molecules. A family of statins have commonly been used during the last several years to treat hypercholesterinaemia and to prevent and treat atherosclerosis of coronary arteries. Mevastatin - a novel inhibitor of HMG-COA reductase, the rate-limiting enzyme of the mevalonate pathway, and mevalonic acid is an essential precursor of isoprenoid compounds including farnesyl isoprenoid, cholesterol etc. There are scattered data, that statins could have antiproliferative effect. Lovastatin has been shown to arrest both tumor and normal cells in the G1 phase of the cell cycle. Two clinical studies showed reduction in the number of newly diagnosed colorectal cancer during a five year follow-up period in patients reciving pravastatin and simvastatin. Moreover, in vitro studies have shown that statins can trigger apoptosis in a variety of tumor cell lines.

Apoptosis is an important mode of cell death. The apoptosis program is switched on either by internal inducers increasing mitochondrial membrane permeability or by death ligand/receptor complex. In both cases the downstream caspase system is the main executor. Apoptosis is a critical process in normal B-cell function, and in many cases the abnormalities in the apoptotic pathways contribute to the pathogenesis of B-cell malignancies. This scenario has particular relevance in multiple myeloma (MM), which is characterized by the continuous accumulation of malignant plasma cells in the bone marrow. It is possible that protectiv mechanisms which inhibit or suppress apoptosis may participate in the induction and maintenance of a malignant MM clone.

Syndecans are a family of cell surface proteoglycans. All four known members of the syndecan family (syndecan 1-4) are type I transmembrane proteins with highly conserved transmembrane and cytoplasmatic domains and an extracellular domain. Syndecan-1 (CD138) is expressed predominantly on epithelial cells but it is also found on various lymphoid cells of the B lineage.
In the bone marrow of myeloma patients syndecan-1 is expressed only on the surface of myeloma cells. Expression can also be detected on the surface of malignant plasma cells entering the peripheral blood. It is believed that syndecan-1 mediates specific adhesion of myeloma cells to type I collagen. It also inhibits the in vitro invasion of malignant plasma cells into type I collagen gels and mediates cell-cell adhesion between myeloma cells. Their heparan sulfate side chains, HSPGs have the ability to bind numerous growth factors. Syndecan-1 is shed from the surface of myeloma cells in culture and inhibits myeloma cell growth and induces apoptosis in vitro.
THE AIM OF THE STUDY

1. The aim of the in vitro study is to determine the effect of mevastatin on proliferation and apoptosis in U266 myeloma cells.

2. In this clinical study, we analyzed the levels of syndecan-1 in sera of patients suffering from MM, solitary plasmacytoma and monoclonal gammopathy of undetermined significance (MGUS). We also compared the serum syndecan-1 level of MM patients stratified by the Salmon-Durie staging system. Finally, we investigated the correlation of serum syndecan-1 levels with other independent prognostic factors of MM such as serum beta2-microglobulin.
MATERIALS AND METHODS

A. In vitro study

Cell culture
Experiments were performed on a human U266 myeloma cell line. Cells were treated with mevastatin in doses of 0.5-1-2.5 μg/ml for 0-96 hours in 24 well plates/flasks in a concentration of 6 x10^5 cells/ml.

Chemicals
Mevastatin (Sigma) was solved in DMSO (10 mg/ml), and was stored at –20°C. To study the role of caspases ZVAD-fmk (Enzym System Product), a general caspase inhibitor, Z-IETD-fmk (Pharmingen), a caspase-8 inhibitor, and Z-LEHD-fmk (Pharmingen), a caspase-9 inhibitor was given in 50-100 μM to mevastatin treated cultures. In the long mevastatin treatment period (72-96 h) the caspase inhibitor was provided in every 24 h.

Cell cycle analysis and apoptosis measurements
Cells were kept for another 15 min before flow cytometric measurements to estimate cell cycle and proportion of sub-G1 (apoptotic) cells. The flow cytometric measurements were made on a FACSCAN flow cytometer.

Western-blot
30 μg of total protein was used for western-blot on a 12.5% polyacrylamide gel and electrotransferred onto PVDF membrane (Bio-Rad). Monoclonal antibodies for Bcl-2, Bid and polyclonal antibody for Bax were used for Western blot analysis. Developing was performed by Vectastain ABC Kit and ECL+Plus chemiluminescens detection kit

RT-PCR
Total RNA was isolated from the 10 x 10^6 cells by Quiagen RNeasy kit using standard procedures. Complementary DNA was synthetized from 100 ng RNA using MMLV reverse transcriptase and random primers. In semiquantitative PCR β-actin was used as a control to monitor amplification efficiency and the quality of the cDNA. Parameters of PCR reaction: 65°C, 1 min, 72°C, 1 min, 28
cycles. In PCR reactions red-Taq polymerase enzyme was used. PCR products were analysed in 1,5% agarose gelelectrophoresis and visualised by ethidium bromide staining; and evaluated by Eagle Eye video densitometer.

*Caspase activity*

The cells were washed in PBS and the caspase-3 activity was detected by fluorogenic substrate, DEVD-AMC in PBS–EGTA buffer (5 mM EGTA, 1 mM DTT, 12,5 mM DEVD-AMC) with excitation at 380 nm, and extension 460 nm. Each sample contained 5x10^5/ml cell. Samples were measured in Fluoroskan Ascent Fluorimeter.

*Depolarisation of mitochondrial membrane*

To detect the mitochondrial membrane depolarization, cells were incubated with DiOC6 (20nM, 3,3-dihexilocarbocyan-iodide) and propidium iodide for 15 min and the changes of fluorescence were measured by flow cytometry (at 530-620 nm).

*Detection of Bax translocation*

The treated and control cells were incubated with CMTMRos (Molecular Probes) (5 ?M) for 15 minutes. After washing with PBS, they were fixed in ice cold 80 % methanol. We used a polyclonal antibody (1:20) specific for Bax and the reaction was developed by Vectastain ABC Kit and Streptavidin–FITC. Samples were examined by confocal microscope.

*TRAIL, TNF-alfa, FASL neutralization*

The study the role of autocrine TRAIL, TNF-alfa, FASL in the mevastatin induced apoptosis the potential effect of these death ligands were neutralized using recombinant TRAIL-R2Fc (100ng/ml), monoclonal anti-human TNF-alfa (1 ng/ml) or NOK-1 (anti-FASL antibody, 1 ng/ml).

*Syndecan-1 measurement by flow cytometry*

Cells were fixed in ice cold -80% methanol and incubated with anti-syndecan antibody (MCA681, 1:200). The reaction was developed with biotinylated anti-mouse antibody for 45 min followed by washing and subsequent addition of avidin-FITC. Cells were analysed using FACSCAN flow cytometry.
B. In vivo study

Study population
A total of 67 patients entered the study, 13 with MGUS, 4 with solitary plasmocytoma, and 50 with MM. All plasma cell dyscrasias were defined by the diagnostic criteria of the American Southwest Oncology Group (SWOG).

Serum syndecan-1 analysis
Serum concentration of soluble syndecan-1 was measured using a commercially available human syndecan-1 ELISA assay kit.
RESULTS

A. In vitro study

Effect of mevastatin on U266 cells:
Mevastatin inhibited cell proliferation and induced apoptosis in U266 cells. Proliferation was blocked mainly in G1 phase indicated by the increase of cells with G1 DNA-content. The mevastatin induced apoptosis was time (24-96 h) and dose (1-2.5 \( \mu \)g/ml) dependent. The apoptotic response was relatively slow, appearing after 48 h and reaching the highest values at 72-96 h.

The mechanism of the induced apoptosis:
The role of caspases was proved by the inhibitory effect of Z-VAD-fmk on the mevastatin induced apoptosis in U266 cells. Furthermore, caspase-3 is a key protease in the late phase of the apoptosis. Mevastatin treatment was followed by an increase of the cleaved colorimetric substrate of caspase-3, indicating that caspase-3 was activated. Apoptosis was also inhibited in cells pretreated with caspase-8 inhibitor or caspase-9 inhibitor.
Using DiOC6 staining a dose dependent depolarization of the mitochondrial membrane was found at 48 h after mevastatin treatment cells. Among the factors regulating mitochondrial membrane permeability the expression of Bcl-2 mRNA was downregulated in U266 cells, but the expression of Bax and Bcl-X\(_L\) was not influenced. The Western blot analysis supported the decreased expression of Bcl-2 protein. The expression of proapoptotic Bax protein was not changed, however the Bcl-2/Bax ratio was decreased and the location of Bax protein was also changed. After 72 h mevastatin treatment confocal microscopy suggested that the homogenous cytoplasmic Bax staining was replaced by a bright mitochondrial staining. We studied the cleavage of Bid by Western blot analysis: the detectable fragment of Bid decreased at 72 h after mevastatin treatment.
The potential role of the death-ligands (TRAIL, TNF\(_\alpha\), FasL) in the mevastatin induced apoptosis was checked by neutralization experiments. None of the neutralizing antibodies (TRAIL-R2Fc, anti-TNF\(_\alpha\), Nok1) influenced the effect.
of mevastatin, suggesting that the change in caspase-8 activity is independent from death-receptors.

Syndecan-1 shedding:
There are observations that apoptosis of myeloma cells could be accompanied by the shedding of cell surface syndecan-1. Our results further supported this finding: flow cytometric analysis showed that the level of cell surface syndecan-1 expression and the number of syndecan-1 positive cells was time dependently reduced during mevastatin induced cell death.

B. In vivo study

Serum concentrations of syndecan-1:
The serum syndecan-1 concentrations in patients with MGUS, solitary plasmocytoma, and MM at the time of the diagnosis. Mean serum syndecan-1 concentrations of patients with MGUS (n=13) and solitary plasmocytoma (n=4) were 77.9 ng/mL (range: 33-122) and 65.6 ng/mL (range: 33.8-94.5), respectively. In a contrast to these values median syndecan-1 concentration in the group of MM patients (n=50) was 223.8 ng/mL (range: 36-508). Although the number of the MGUS patients and those in the solitary plasmocytoma group was low the differences between the median syndecan-1 values were statistically significant.

Statistically significant differences of serum syndecan-1 concentrations in Salmon-Durie subgroups of MM patients could also be detected. Parallel to the increase of tumor load, there is also an increase in the serum syndecan-1 level of patients.

Correlation of serum syndecan-1 concentration with other prognostic factors in MM patients:
Correlation was found with the serum creatinine and beta2-microglobulin levels, the monoclonal protein concentration, and the bone marrow plasma cell count.
Follow up of MGUS and MM patients:
Over the course of a 6 months follow up period there was no increase in the serum syndecan-1 level in clinically stable MGUS patients.
15 MM patients were followed after initiation of chemotherapy. Seven patients received chemotherapy according to the ICOMP protocol, whereas 8 patients were given the classic VAD regimen. Additional bisphosphonate treatment was also introduced for all 15 patients. Baseline serum syndecan-1 levels were determined before initiation of chemotherapy and follow up syndecan-1 levels were measured after 6 months. A significant decrease in the median syndecan-1 level was seen in patients who responded to chemotherapy. In contrast, there was no change in serum syndecan-1 levels of non responder patients.
DISCUSSION

It has recently been found that statins can induce apoptosis in variety of malignant cells.

In this in vitro study, we have found that mevastatin induced cell cycle arrest and apoptosis in U266 human myeloma cells. Apoptosis induction was dependent on caspase-3 activity, which is a crucial step in almost all apoptotic response. It seems that both caspase-8 and caspase-9 took part in caspase activation, but the extrinsic (death receptor) and intrinsic (mitochondrial) apoptotic pathways reacted differently. (The involvement of caspases was proved by the use of different caspase inhibitors.)

Caspase-8 could be activated either by death receptors or by other caspases. In U266 cells none of the death ligand neutralizing antibodies – TRAIL-R2Fc, anti-TNFα, and anti-FasL-Nok-1 - influenced the mevastatin caused apoptosis. Therefore the second option is highly possible, i.e. caspase-8 was activated by other caspases (e.g. caspase-3) through the activity of the mitochondrial pathway. In that case caspase-8 serves as a helper to speed up apoptosis by cleaving Bid, which induce the release of cytochrome c from mitochondria. Cytochrom c in complex with Apaf-1 activates caspase-9 which in turn activates the executioner caspase.

Many data of our study supported that mevastatin acts primarily through the intrinsic apoptotic machinery. Using DiOC6 it was found that the depolarization of mitochondrial membrane increased at 48 h after treatment. Expression of Bcl-2 (both mRNA and protein) decreased, while Bax was translocated from the cytosol to the mitochondria showed by confocal microscopy. Finally, the caspase-9 inhibitor blocked the mevastatin caused apoptosis. The time-course of apoptosis - the relatively slow start and speeding up later - also support the early involvement of the mitochondrial components with a gradual help of caspase-8.

We also found that mevastatin induced apoptosis was accompanied by a reduced the level of cell surface syndecan-1 in U266 cells. It is highly possible that syndecan-1 shedding and its appearance in the circulation could be a clinical marker for apoptosis in myeloma cells.
It is unrealistic that statins can be used as single antitumor agents. However, the potentiation of antitumor activity of the existing chemotherapeutic regimens by statins could extend the therapeutic efficiency of such combinations by opening up or inducing apoptotic response. This view is supported by our findings with mevastatin in myeloma cells.

High soluble syndecan-1 level in serum is associated with poor prognosis in lung and head and neck cancers. Previous studies have shown that syndecan-1 is shed from the surface of myeloma cells into serum. Measured by a semiquantitative method, syndecan-1 levels were elevated in the sera of 7 out of 20 myeloma patients. Higher soluble serum syndecan-1 levels were associated with higher levels of serum beta₂-microglobulin and elevated plasma cell count in the bone marrow. Evaluation of data collected from 138 MM patients showed that the serum syndecan-1 value could serve as an independent prognostic parameter in addition to serum beta₂-microglobulin and WHO performance status. From a pathophysiologial standpoint it has to be emphasized that soluble syndecan-1 is present at high concentrations in the bone marrow of patients with MM and shed syndecan-1 accumulates within the fibrotic regions of bone marrow. As cell surface syndecans have been implicated in several important biologic processes such as cell adhesion and migration and intercellular and cell-matrix interactions, this phenomenon may contribute to the homing mechanism of transformed plasma cells to the bone marrow. Another aspect is that the accumulation of syndecan-1 in the bone marrow may lead to a local enrichment of bound growth factors. Recently, it has been shown that cell surface syndecan-1 mediates hepatocyte growth factor binding and promotes Met signaling in MM.

In our study, patients with MM showed higher median level of serum syndecan-1 than patients with MGUS and plasmocytoma. The differences between these groups were significant. A comparison of serum syndecan-1 among the myeloma subgroups also revealed significant differences. Correlation was found between the level of serum beta₂-microglobulin, monoclonal protein concentration or bone marrow plasma cell count. A significant decrease in median syndecan level was observed in patients responding to chemotherapy, whereas median syndecan level did not change in nonresponders. It is also notable that we found low levels of circulating syndecan-1 in two patients with nonsecretory MM. One may presume that there might be a more intricate
relationship between immunoglobulin secretion and syndecan-1 expression. In addition to this, our data draw attention to the strong correlation of serum creatinine and syndecan-1 and beta$_2$-microglobulin levels. It is also conceivable that in patients with poor renal function a parallel accumulation of beta$_2$-microglobulin and syndecan-1 may occur. Further investigations are needed to explore this problem.
SUMMARY

A.
-Mevastatin inhibited cell proliferation and induced apoptosis in U266 cells.
-Mevastatin treatment was followed by an increase of the cleaved colorimetric substrate of caspase-3, indicating that caspase-3 was activated.
-Apoptosis was also inhibited in cells pretreated with caspase-8 inhibitor or caspase-9 inhibitor.
-Using DiOC6 staining a dose dependent depolarization of the mitochondrial membrane was found at 48 h after mevastatin treatment cells.
-The expression of Bcl-2 mRNA was downregulated in U266 cells, but the expression of Bax and Bcl-XL was not influenced.
-The Western blot analysis supported the decreased expression of Bcl-2 protein.
-The expression of proapoptotic Bax protein was not changed, however the Bcl-2/Bax ratio was decreased and the location of Bax protein was also changed.
-None of the neutralizing antibodies (TRAIL-R2Fc, anti-TNF?, Nok1) influenced the effect of mevastatin, suggesting that the change in caspase-8 activity is independent from death-receptors.

B.
-MM showed higher median level of serum syndecan-1 than patients with MGUS and plasmocytoma
-Statistically significant differences of serum syndecan-1 concentrations in Salmon-Durie subgroups of MM patients could also be detected. Parallel to the increase of tumor load, there is also an increase in the serum syndecan-1 level of patients.
-Correlation was found with the serum creatinine and beta2-microglobulin levels, the monoclonal protein concentration, and the bone marrow plasma cell count.
-A significant decrease in median syndecan level was observed in patients responding to chemotherapy, whereas median syndecan level did not change in nonresponders.


