Zoledronic Acid Inhibits the Motility of Cancer Stem-like Cells from the Human Breast Cancer Cell Line MDA-MB 231

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Abstract. Background/Aim: Various effects on tumor cells have been described for zoledronic acid (ZOL). However, only limited data exist regarding its influence on the motility of tumor cells. Since migration of tumor stem cells is a decisive step in metastasis, we examined whether ZOL reduces their motility. Materials and Methods: We investigated the effects of ZOL on stem-like progenitor cells obtained via the formation of spheroids from the human breast cancer cell line MDA-MB 231. Stem cell properties were verified by measurement of high CD44 expression and absence of CD24 expression. Motility was explored by time-resolved videography, protein expression by western blotting. Results: ZOL strongly reduced the migration of stem-like progenitor cells. Cellular velocity was reduced by 61% following exposure to 1 μM ZOL and by 82% after exposure to 10 μM ZOL. Accumulated distance traveled by the cells was reduced by 60% and 79% after exposure to 1 μM and 10 μM ZOL, respectively. The remaining cellular motility led to very little change in distance, with cellular activity appearing more as “stepping on the spot”. The reduced motility might be due to reduced phosphorylation of focal adhesion kinase (FAK), an important enzyme in cellular migration. Conclusion: ZOL reduces the motility and cellular velocity of breast cancer cells in vitro. The reduced mobility might slow down or even stop metastasis, which is a known result of adjuvant ZOL therapy in breast cancer patients. In vivo studies are warranted to evaluate the impact of the reduced motility on the metastatic cascade.

Over the past two decades, several effects have been described for zoledronic acid (ZOL). It is well-known that ZOL decreases bone loss and the risk of skeletal fractures in patients with bone metastases (1-3). The classical model of the effect of bisphosphonates (ZOL is one of the most effective agents in this class) suggests that tumor-affected bone harbors a vicious circle of mutual activation of tumor cell and osteoclasts leading to accelerated loss of bone substance and, thus, to an increase in bone fractures or deformities of bone structure (4). Bisphosphonates break this cycle by inhibiting and destroying activated osteoclasts (5, 6). In addition, there is growing experimental evidence that ZOL exerts anti-tumor and anti-metastatic effects (7, 8); inhibition of proliferation, S-phase arrest, invasiveness, neoangiogenesis and induction of apoptosis have been described for various cultured tumor cells (9-11).

Various characteristics of tumor cells contribute to the malignancy of cancer cells, to their metastatic performance. Cell-cell adhesion must be weakened by de-differentiation until the cells are able to leave the epithelial network; the cells must be capable of migration. Finally, the cells need invasive potential in order to cut a path through the connective tissue and into the vasculature. It is known that ZOL exerts antiproliferative effects by activating cellular apoptosis, probably by reducing available energy through inhibition of farnesyl transferase (12) and/or suppression of endogenous topoisomerase II activity, which is associated with apoptosis and S-phase arrest in tumor cells respective cells (13).

Although ZOL is also known to reduce the invasiveness of cancer cells (14), very little is known about the interaction of ZOL with the migratory potential of the cells that is crucial for metastasis (15, 16). Thus, this study was performed to evaluate the effect of ZOL on the migration of cancer cells.

Materials and Methods

Cell culture and isolation of a tumor stem cell fraction. The tumor stem cell hypothesis (17, 18) postulates that recurrences arise from tumor cells with stem cell properties rather than from “normal” somatic tumor cells. It seems plausible that an isolated fraction of
these cells is the best model system for in vitro studies. We, therefore, isolated a fraction of progenitor cells with stem cell properties from the human breast cancer cell line MDA-MB 231 (ATCC, Rochester, NY, USA).

The cells were cultivated in an incubator with 5% CO₂ in air at 37°C. The culture medium was RPMI supplemented with 10% fetal calf serum, penicillin 10 IU/ml and streptomycin 10 μg/ml (all from Biochrom, Berlin, Germany). This cell line is reported to contain tumor stem cells or stem-like progenitor cells in a proportion of at least 15% (19). This fraction was enriched by the formation of spheroids until homogeneity. Aggregates of viable suspension cells in the supernatant over the cell monolayer were harvested and brought into hanging drops until spheroids were formed. The spheroids were dissociated, transferred to a new culture flask, brought to confluence and the spheroids were captured again. This cycle was repeated until the obtained cells were homogenously CD44-positive and CD24-negative.

Immunofluorescence. CD24 and CD44 levels were detected by immunofluorescence. The cells to be investigated were cultivated to 30-50% confluence in Falcon chamber slides (Becton Dickinson, Heidelberg, Germany), fixed with ice-cold 47:47:6 methanol: acetone:formaldehyde for 15 min, washed with phosphate-buffered saline and incubated for 1 h at room temperature with primary murine antibodies against the two surface proteins (Cell Signaling/ New England Biolabs, Frankfurt, Germany), fixed with ice-cold 47:47:6 methanol:acetone:formaldehyde for 15 min, washed with phosphate-buffered saline and incubated for 1 h at room temperature with primary murine antibodies against the two surface proteins (Cell Signaling/ New England Biolabs, Frankfurt, Germany), and documented and analyzed with a ChemiDoc system (Bio-Rad, München, Germany). The cells were incubated for 30 min at room temperature with a biotinylated secondary rabbit anti-mouse antibody (Jackson Labs, Newmarket, UK) and subsequently stained with a streptavidin-Cy3 conjugate (Jackson Labs). Between all staining steps, the cells were washed carefully three times with phosphate-buffered saline plus 0.05% Tween for 5 min.

Incubation with ZOL. A total of 10⁴ cells per well were plated in 6-well plates (Sarstedt, Nümbrecht, Germany). ZOL (Enzo Life Sciences, Lörach, Germany) in RPMI was prepared as a stock solution and filtered for sterility. After one day of culture, ZOL was added to a final concentration of 0, 1 or 10 μM and the cells were incubated for 24 h. Subsequently, the motility of the cells was observed in duplicate and documented by videography over a period of 24 h. In additional experiments, the influence of a ZOL gradient was determined in μ-Slide Chemotaxis chambers (ibidi, München, Germany). According to the manufacturer’s instructions, 2×10⁵ cells were seeded in each of the three independent chambers of the slide. After 3 h, non-adherent cells were carefully washed away and 1 μM ZOL in culture medium was applied to one side of two chambers. The opposite reservoirs and the remaining chamber were filled with medium alone. The slide was kept in the incubator for 1 h to allow homogenous formation of the gradient and then transferred to the videography system to observe and document the motility of the cells.

Videography. Our home-made videography system is based on the Zeiss Axiovert 25 (20). A plate holder, which moves in three dimensions via three precise linear motors, houses 6-well plates and maintains them at 37°C in a constant atmosphere of 5% CO₂ in air. Points of interest can be marked; the system approaches them at selectable time intervals and takes a photograph that is stored electronically. The image stacks were tracked with ImageJ 1.47 (free download at: http://rsbweb.nih.gov/ij/) and analyzed with the ibidi Chemotaxis and Migration Tool 2.0 (free download at: http://ibidi.com/software/chemotaxis_and_migration_tool/). At least 20 cells were tracked for each well.

Image analysis. The mean accumulated distances, Euclidean distances and velocities of three independent experiments were calculated. The respective highest measurements from each well were analyzed as well. Means ± standard errors of the mean are presented for all measurements. Statistics were calculated with GraphPad Prism 5 (GraphPad Software; http://www.graphpad.com/). For the experiments with the ZOL gradient, the coordinates of the center of mass in the direction of the gradient were calculated, as were the distributions of the track end points with respect to the gradient.

Western blots. Focal adhesion kinase (FAK), as well as the phosphorylated enzyme, were quantified by immunoblots. Cells were incubated for 24 h with ZOL, solubilized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (roti-load; Roth, Karlsruhe, Germany), separated on a 10% polyacrylamide gel, blotted on a nitrocellulose membrane and probed with antibodies against FAK or pFAK and β-actin as internal standard (all antibodies from Abcam, Cambridge, UK). The protein bands were visualized with a Lumilight kit (Roche Diagnostics, Mannheim, Germany) and documented and analyzed with a ChemiDoc system (Bio-Rad, München, Germany).

Results

The stem cell properties of the obtained spheroidal fraction of the MDA-MB 231 cell line were confirmed by measuring the CD44/CD24 ratio. Consistent with descriptions of progenitor cells of breast cancer origin (21), the isolated cells exhibited strong, membrane-associated CD44 expression, whereas CD24 was generally not detectable by immunofluorescence (Figure 1). Only one small cell cluster was found to be CD24-positive, indicating the high purity of the tumor stem cell fraction. This positive cluster is shown in Figure 1 to serve as an internal control.

Cell motility was observed and documented over 24 h by time-resolved videography. ZOL was added at concentrations of 0, 1 or 10 μM. While the untreated cells exhibited distinct migration, the addition of 1 μM ZOL caused a strong inhibition of motility (Figure 2). Increasing the ZOL concentration to 10 μM enhanced this effect but substantial inhibition of the migration was observed at 1 μM ZOL (Figure 2). The starting points of the observed cells (n=20 cells for each ZOL concentration) were merged in the origin to clarify the patterns of migration (Figure 2).

Three frames from the videography stack are shown in Figure 3 to demonstrate the marginal motility in the presence of 10 μM ZOL. This motility is less a migration across than a "stepping on the spot." Few cells covered a noteworthy distance (marked “b” in Figure 3). Apoptosis was not observed. Cell divisions (marked “a” in Figure 3) indicate that the inhibition of the cellular motility was specific, and not due to a general, unspecific toxic effect of ZOL.
Figure 1. Progenitor cells exhibit homogenous, high CD44 expression (up) and almost no expression of CD24 (down). Progenitor cells were isolated from spheroids of the human breast cancer cell line MDA-MB 231; CD44/CD24 expression was measured via immunofluorescence. Very few cells remained CD24-positive. This section served as an internal standard for CD24 staining.

Figure 2. Cellular migration following incubation with 0, 1 or 10 μM ZOL. Cell motility was documented by time-lapse videography. The cells were tracked using ImageJ and analyzed with the Chemotaxis and Migration Tool provided by ibidi. Example plots of ~20 tracked cells per condition are shown. The starting points of the tracks are merged into the origin to highlight the migration patterns. Cellular migration decreased as the concentration of ZOL increased.
The values of several motility parameters were markedly reduced in the presence of ZOL (Figure 4). The mean velocity of the cells dropped from 8.64±0.53 μm/h in the presence of 0 μM ZOL to 3.46±0.19 and 1.52±0.10 after exposure to 1 μM and 10 μM ZOL, respectively. The mean accumulated distance declined from 175.7±10.7 μm traveled in 24 h (0 μM ZOL) to 70.6±3.9 μm and 36.9±2.3 μm, respectively. In addition to the exact route taken by the cells, which often switched orientation, we also considered the Euclidean distance traveled by the cells. This translocation describes the upright distance between the starting point and the end point being, thus, a measure for the real gain in distance from the origin. After 24 h, the mean Euclidean distance traveled by the untreated control cells was 30.3±4.0 μm. ZOL reduced this translocation to 16.0±2.0 μm at 1 μM and to 8.3±0.9 μm at 10 μM.

The maximal values for velocity were 15.6±3.2 μm/h (0 μM ZOL), 5.6±0.5 μm/h (1 μM ZOL) and 2.9±0.1 μm/h (10 μM ZOL), while we observed accumulated distances of 317.5±65.0 μm (0 μM ZOL), 118.1±13.4 μm (1 μM ZOL) and 71.1±3.2 μm (10 μM ZOL). For Euclidean distance, the measurements were 104.1±5.4 μm (0 μM ZOL), 49.8±7.8 μm (1 μM ZOL) and 22.8±7.7 μm (10 μM ZOL).

Application of a gradient from 0 to 1 μM ZOL revealed that the mean direction of cellular migration turned away from increasing ZOL concentrations (Figure 5); of 60 analyzed tracks, 46 were oriented away from ZOL. The center of mass of the end points was shifted 26.8 μm away from the origin. For control cells grown without a ZOL gradient, 19/40 tracks presented in the same direction with a center of mass shifted 0.6 μm in the same direction (data not shown).

FAK is an important enzyme in cellular migration. It links integrins to the cytoskeleton and to various signal transduction pathways. The enzyme is activated by phosphorylation at tyrosin residues. In order to clarify whether the reduced motility after ZOL incubation is due to a less active FAK, the enzyme, as well as its phosphorylated fraction, were analyzed by western blotting. Figure 6 reveals that the expression level increased slightly with increasing concentrations of ZOL, whereas the phosphorylated part was significantly and dose-dependently reduced. Normalized values for 0, 0.5, 1 and 10 μM ZOL for total FAK expression were: 100±5.7, 105.8±4.2, 130.6±11.3 and 107.4±6.6. For the phosphorylated fraction: 100±6.8, 92.8±9.3, 60.7±5.1 and 48.3±6.1.

**Discussion**

The present investigation demonstrates that ZOL strongly limits the motility of breast cancer progenitor cells. The mean values, as well as the highest values, for velocity, accumulated distance and Euclidean distance were heavily reduced. In a ZOL gradient, the mean direction of cellular migration was oriented away from higher ZOL concentrations. This observation does probably not reflect a chemotactic effect but indicates a slowdown that increases in parallel with increasing concentrations of the inhibitor.
Figure 4. ZOL influences the motility parameters velocity, accumulated distance and Euclidean distance. Cellular motility in the presence of 0, 1 or 10 μM ZOL was documented by time-lapse videography over 24 h. The cells were tracked using ImageJ and analyzed with the chemotaxis and migration tool provided by ibidi. Mean values from at least 100 cells are depicted on the left and the mean of the highest values from six individual data sets appear on the right. All parameters were reduced in a dose-dependent manner. Error bars: S.E.M. Levels of significance (Mann-Whitney U-Test): *** if p≤0.001; **** if p≤0.0001.
ZOL seems to counteract metastasis at many levels, including inhibition of proliferation (22) and invasiveness (23). A 2009 study reported an improvement in disease-free survival (DFS) following the addition of ZOL to an adjuvant endocrine breast cancer therapy in premenopausal estrogen receptor (ER)-positive patients (24). ZOL not only reduced bone metastasis, as expected, but also reduced visceral and overall metastasis. Other recent studies showed similar findings on DFS and overall survival (25, 26), while other investigations observed no benefit following the addition of ZOL (27, 28). It seems that only some subgroups of breast-cancer patients with tumor characteristics that have not been completely defined may benefit from ZOL treatment.

The in vitro results presented here hint at the mechanism underlying the benefit of ZOL. The ability of tumor cells to migrate was greatly impaired by concentrations of ZOL that are therapeutically achievable.

Systemic metastasis in breast cancer is assumed to be a two-step process. Even in early stages of the disease, micrometastases may settle in the bone marrow where they can remain inactive for long periods of time (29, 30). Motility is one the crucial factors determining the eager to metastases of cancer cells, particularly, in a "fluid", well-vascularized environment as the bone marrow. One should note that the bone marrow has no rigid connective tissue that

Figure 5. Migrating cells trend away from a gradient of 0-1 μM ZOL. ZOL was applied to cells in μ-Slide Chemotaxis chambers and cellular motility was documented by time-lapse videography over 24 h. The cells were tracked using ImageJ, analyzed with the chemotaxis and migration tool provided by ibidi and compared to controls that were not exposed to the ZOL gradient (data not shown). The depicted example shows tracks and end points from 30 cells (left). The starting points are merged at the origin. Cells with end points on lower ZOL concentrations are plotted in purple and cells with end points on higher ZOL concentrations appear in green. The center of mass is depicted in light blue. The rose plot of the frequency of cellular presence in specified sectors of the area (right) also reveals that migrating cells avoid the area of high ZOL concentration.

Figure 6. ZOL induces slightly the expression of FAK but reduces its phosphorylation-dependent activation. Expression and phosphorylation of FAK after incubation with 0, 0.5, 1 or 10 μM ZOL for 24 h. The cells were solubilized with sample buffer, proteins separated by PAGE, blotted and probed with antibodies against FAK, pFAK and β-actin (internal standard). Normalized means from 3 single experiments. Error bars are S.E.M.
would prohibit the travelling cells from penetration. Thus, invasiveness plays, most probably, a minor role in further spread of the disease in the bone marrow. Direct observation by videography is a method that enables an excellent evaluation of cell motility without the disadvantages of experiments in transwell chambers that provide mixed information on migration capability and invasiveness.

Of further interest would be an accurate analysis of all aspects of cellular motility, not only locomotion. We observed that, in the presence of ZOL, many cells stopped migrating but were not completely immobile. The membranes of these cells were always in action, often moving rhythmically back and forth; we describe this action as a "stepping on the spot". Sometimes, small displacements of the nucleus by no more than one cell width were observed, often on a small circular path (data not shown). It would certainly be of interest to characterize this "micro-motility" and to correlate it with the malignancy of the tumor cells.

Re-differentiation is the last determining factor on the aggressiveness of tumor cells that remains uncharacterized. Generally, an epithelial-mesenchymal transition occurs during malignant transformation. ZOL exposure may induce a reversion, a mesenchymal-epithelial transition, resulting in less aggressive micrometastases in the bone marrow. This hypothesis is currently under investigation in our laboratory.

ZOL is known to inhibit farnesyl transferase and, thus, impair mevalonate metabolism (31), significantly reducing the number of available intracellular energy equivalents. Therefore, the reduction in motility following ZOL exposure may be due, at least in part, to energy depletion. However, we observed that the cells were still able to divide in the presence of 10 μM ZOL (Figure 3); a depletion of energy that stops migration would probably also disrupt mitosis.

First attempts to clarify the underlying mechanism of ZOL action revealed that the phosphorylation of FAK is dose-dependently reduced after 24 h of incubation. FAK as important promoter of cellular migration is activated by phosphorylation. The less active enzyme after ZOL incubation might be responsible for the reduced motility observed in the tracking experiments. The increase in total FAK expression might be due to back coupling loops induced by the loss of FAK activity.

To our knowledge, this is the first study using direct videography that reports the impact of ZOL on cell motility. Detailed studies on the processes that underlie the observed changes in the motility due to ZOL exposure of tumor cells are currently designed.

Conclusion

Our results indicate that zoledronic acid reduces the motility and cellular velocity of breast cancer stem-like cells in vitro. This might slow-down or even stop the metastatic cascade and may hint at the mechanism underlying the known clinical benefit of ZOL. Further experimental and clinical studies are warranted to evaluate the impact of the reduced motility on the metastatic cascade.

Disclosure

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