Amygdalalin blocks the in vitro adhesion and invasion of renal cell carcinoma cells by an integrin-dependent mechanism

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Abstract. Information about the natural compound amygdalin, which is employed as an antitumor agent, is sparse and thus its efficacy remains controversial. In this study, to determine whether amygdalin exerts antitumor effects on renal cell carcinoma (RCC) cells, its impact on RCC metastatic activity was investigated. The RCC cell lines, Caki-1, KTC-26 and A498, were exposed to amygdalin from apricot kernels, and adhesion to human vascular endothelium, immobilized collagen or fibronectin was investigated. The influence of amygdalin on chemotactic and invasive activity was also determined, as was the influence of amygdalin on surface and total cellular α and β integrin expression, which are involved in metastasis. We noted that amygdalin caused significant reductions in chemotactic activity, invasion and adhesion to endothelium, collagen and fibronectin. Using FACScan analysis, we noted that amygdalin also induced reductions, particularly in integrins α5 and α6, in all three cell lines. Functional blocking of α5 resulted in significantly diminished adhesion of KTC-26 and A498 to collagen and also in decreased chemotactic behavior in all three cell lines. Blocking α6 integrin significantly reduced chemotactic activity in all three cell lines. Thus, we suggest that exposing RCC cells to amygdalin inhibits metastatic spread and is associated with downregulation of α5 and α6 integrins. Therefore, we posit that amygdalin exerts antitumor activity in vitro, and this may be linked to integrin regulation.

Introduction

Renal cell carcinoma (RCC) is the most common renal tumor. Approximately one-third of patients have metastases at diagnosis, and up to 30% of patients develop metastases during therapy. Once metastasized, the prognosis for patients is bleak. A better understanding of the molecular modes of action underlying RCC development and progression has contributed to the development of targeted therapies, thus improving the outlook for patients in the advanced stages of this disease. However, despite these therapeutic advances, the prognosis for patients with RCC remains poor, with 5-year survival remaining between 5 and 12%. Dissatisfaction with conventional therapy and the desire to reduce side-effects have led many patients to complementary and alternative medicine (CAM). Up to 80% of cancer patients in the United States, and more than 50% of cancer patients in Europe use CAM alongside, or in place of, conventional therapy.

Information about the efficacy of natural compounds is sparse, and certain of these compounds, such as the cyanogenic diglycoside amygdalin (D-mandelonitrile-β-gentiobioside), remain controversial. Amygdalin is derived from the fruit kernels of the Rosaceae family, which includes Prunus persica (peach), Prunus armeniaca (apricot) and Prunus amygdalus var. amara (bitter almond). Amygdalin, mainly in the United States, has been administered to cancer patients since the 1920s. In the 1950s, an intravenous, chemically different form of amygdalin was synthesized and patented as laetrile. Although laetrile differs from amygdalin, the terms are often used interchangeably, making data interpretation difficult. By 1978 approximately 70,000 cancer patients in the US had been treated with amygdalin. Evidence-based research on amygdalin, however, remains limited. A clinical study sponsored by the National Cancer Institute over 30 years ago revealed no signs of tumor regression (1), whereas a retrospective analysis of 67 tumor patients receiving amygdalin reported two complete and four partial responses (2). Ambivalence is also reflected in case reports: amygdalin was ineffective in five cases, and effective in four. Randomized clinical trials and follow-up studies have not been carried out, to the best of our knowledge. Proponents consider amygdalin an effective natural cancer treatment option, whereas opponents warn of toxicity due to hydrogen cyanide metabolism.

Metastasis is the main cause of RCC-associated mortality. Transendothelial migration and motile spread are critical steps in tumor dissemination and progression (3), and the dissemination of cancer cells to distant organs constitutes the major
clinical challenge in treating cancer. In the present study, the anti-neoplastic effect of amygdalin on RCC cell adhesion and migration properties was investigated. Since integrins activate a number of intracellular signaling pathways involved in cell proliferation, differentiation and motility, the expression pattern of α and β integrin adhesion receptors between amygdalin-treated cells and untreated controls was determined. Integrins are important in both health and disease (4) and play a pivotal role in carcinogenesis and cancer progression (4).

The present study is based on a previous investigation dealing with the influence of amygdalin on the metastatic properties of three bladder cancer cell lines (5). Since some dissimilarities were observed regarding the action of amygdalin on the metastatic properties of the different bladder cancer cell lines, the question arose as to whether the different effects of amygdalin were restricted to particular tumor entities or occur in others. Thus, three RCC cell lines were chosen, since RCC tumors are the most aggressive urologic tumor.

Materials and methods

Cell culture. Kidney carcinoma cells, Caki-1, KTC-26, and A498, were purchased from LGC Promochem GmbH (Wesel, Germany). The cells were grown and subcultured in RPMI-1640 medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES buffer, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO₂. Subcultures from passages 5-24 were selected for experimental use. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins by enzyme treatment with dispase (1 U/ml; Gibco-Invitrogen, Carlsbad, CA, USA). HUVECs were grown in Medium 199 (M199; Biozol, Munich, Germany), supplemented with 10% FCS, 10% pooled human serum, 20 µg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin, 100 ng/ml gentamycin and 20 mM HEPES buffer (pH 7.4). Subcultures from passages 1-5 were selected for experimental use. The Institutional Ethics Committee of Goethe-University Hospital, Frankfurt, Germany, waived the need for consent since HUVECs were used anonymously for in vitro assays and had no links with patient data.

Amygdalin treatment. Amygdalin from apricot kernels (Sigma-Aldrich, Taufkirchen, Germany) was freshly dissolved in cell culture medium and then added to and left on the HUVEC monolayer for 1, 2 or 4 h. Subsequently, non-adherent tumor cells were washed off using warmed (37°C) PBS (Ca²⁺ and Mg²⁺). The remaining cells were fixed with 1% glutaraldehyde. Adherent tumor cells were counted in five different fields of a defined size (5x0.25 mm²) using a phase contrast microscope (ID03, 471202-9903; Carl Zeiss Microscopy GmbH, Goettingen, Germany), and the mean cellular adhesion rate was calculated.

Attachment to immobilized extracellular matrix proteins. The 24-well plates were coated with collagen G (extracted from calfskin, consisting of 90% collagen type I and 10% collagen type III, and diluted to 400 µg/ml in PBS; Biochrom, Berlin, Germany) or fibronectin (extracted from mice and diluted to 100 µg/ml in PBS; Becton-Dickinson, Heidelberg, Germany) overnight. Plastic dishes served as the background control. Plates were washed with 1% bovine serum albumin (BSA) in PBS to block non-specific cell adhesion. Tumor cells (0.1x10⁶) were then added to each well and left for 30 min incubation. Subsequently, non-adherent tumor cells were washed off, the remaining adherent cells were fixed with 2% glutaraldehyde and counted microscopically. The mean cellular adhesion rate, defined by adherent cellsadherent cellsbackground was calculated from five different observation fields.

Chemotactic activity. Serum-induced chemotactic movement was examined using 6-well Transwell chambers (Greiner, Frickenhausen, Germany) with 8-µm pores. The cells (0.5x10⁶ Caki-1, KTC-26 or A498 cells/ml) were placed in the upper chamber in serum-free medium, either free of amygdalin (control) or containing amygdalin. The lower chamber contained 10% serum. Following overnight incubation, the upper surface of the transwell membrane was gently wiped with a cotton swab to remove cells that had not migrated. Cells moving to the lower surface of the membrane were stained with hematoxylin and counted microscopically. The mean migration rate was calculated from five different observation fields.

Invasion. Invasion was examined by serum-induced chemotactic movement through a membrane (Greiner) with 8-µm pores, pre-coated with collagen G (extracted from calfskin, consisting of 90% collagen type I and 10% collagen type III; diluted to 400 µg/ml in PBS; Biochrom) and HUVECs, grown to confluence. Caki-1, KTC-26 or A498 cells (0.5x10⁶/ml) were placed in the upper chamber in serum-free medium, either free of amygdalin (control) or containing amygdalin. The lower chamber contained 10% serum. After overnight incubation, the upper surface of the transwell membrane was gently wiped with a cotton swab to remove cells which had not migrated. Cells which had moved to the lower surface of the membrane were stained using hematoxylin and counted microscopically. The mean migration rate was calculated from five different observation fields.

Integrin surface expression. Tumor cells were washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min at 4°C with phycoerythrin (PE)-conjugated monoclonal antibodies directed against the following integrin subtypes: anti-α1 (mouse IgG1; clone SR84; #559596), anti-α2 (mouse IgG2a; clone 12F1-
Western blotting. To investigate integrin content, tumor cell lysates were applied to a 7-12% polyacrylamide gel (depending on protein size) and electrophoresed for 90 min at 100 V. The protein was then transferred to nitrocellulose membranes. After blocking with non-fat dry milk for 1 h, the membranes were incubated overnight with the following antibodies: integrin α1 (rabbit, polyclonal, 1:1,000; #AB1934; Chemicon/Millipore GmbH, Schwalbach, Germany), integrin α2 (mouse IgG1, 1:250, clone 2; #61017; BD Biosciences), integrin α3 (rabbit, polyclonal, 1:1,000; #AB1920; Chemicon/Millipore GmbH), integrin α4 (mouse, 1:200, clone: C-20; #sc-6589; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), integrin α5 (mouse IgG2a, 1:5,000, clone 1; #610634; BD Biosciences), integrin α6 (rabbit, 1:200, clone H-87; #sc-10730; Santa Cruz Biotechnology, Inc.), and integrin β1 (mouse IgG1, 1:2,500, clone 18; #610468), integrin β3 (mouse IgG1, 1:2,500, clone 1; #611141) and integrin β4 (mouse IgG1, 1:250, clone 7; #611233) (all from BD Biosciences). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (both 1:5,000; Upstate Biotechnology, Lake Placid, NY, USA) served as the secondary antibodies. Additionally, integrin-related signaling was explored by anti-integrin-linked kinase (ILK) (clone 3, dilution 1:1,000; #611803), anti-focal adhesion kinase (FAK) (clone 77, dilution 1:1,000; #610088) and anti-p-specific FAK (pY397; clone 18, dilution 1:1,000; #61807) antibodies (all from BD Biosciences). HRP-conjugated goat-anti-mouse IgG (dilution 1:5,000; Upstate Biotechnology) served as secondary antibodies. The membranes were briefly incubated with ECL detection reagent (ECL™; Amersham, GE Healthcare, München, Germany) to visualize the proteins and then analyzed with the Fusion FX7 system (Peqlab, Erlangen, Germany). β-actin (1:1,000; Sigma-Aldrich) served as the internal control.

Gimp 2.8 software was used to perform pixel density analysis of the protein bands. The ratio of protein intensity/β-actin intensity was calculated, and expressed as a percentage, related to controls set to 100%.

Blocking experiments. To determine whether integrin α5 and α6 impacted the metastatic spread independently of amygdalin in Caki-1, KTC-26, and A498 cell lines, cells were incubated for 60 min with 10 µg/ml function-blocking anti-integrin α5 (clone P1D6) mouse mAb or anti-integrin α6 (clone NKI-GoH3) rat mAb (both from Millipore). Controls were incubated with cell culture medium alone. Subsequently, tumor cell adhesion to immobilized collagen as well as chemotaxis were analyzed as described above.

Statistical analysis. In the present study, all experiments were performed 3-6 times. Statistical significance was determined using the Wilcoxon, Mann-Whitney U-test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Amygdalin blocks interaction between the tumor cell endothelium and tumor cell matrix. After 24 h of treatment with amygdalin, A498 tumor cell adhesion to HUVECs was significantly diminished, but cell adhesion of Caki-1 and KTC-26 cells was not (compared to untreated controls set to 100%) (Fig. 1). Extending the exposure time of amygdalin to 2 weeks significantly decreased adhesion to HUVECs in all three tumor cell lines (Fig. 1).

Amygdalin caused a significant decrease in the binding capacity of all three RCC cell lines to immobilized collagen.
and fibronectin, compared to controls (Fig. 2). Attachment of all three cell lines to the matrix proteins was diminished after 24 h, as well as after 2 weeks. In the KTC-26 cell line, short-term amygdalin application (24 h) induced a greater decrease in adhesion than long-term amygdalin application (2 weeks).

**Amygdalin alters the tumor cell motility of RCC cells.** The chemotactic activity of Caki-1, KTC-26 and A498 cells significantly decreased after 24 h and 2 weeks of amygdalin application, compared to the untreated control cells (Fig. 3A). Tumor cell invasion through the collagen-coated transwell membranes was also significantly diminished in Caki-1 and A498 cells after 24 h and 2 weeks of amygdalin application (Fig. 3B). However, 2 weeks of amygdalin did not reduce the invasive capacity of KTC-26 cells.

**Amygdalin modulates integrin α and β surface expression.** Caki-1, KTC-26 and A498 cells were characterized by different basal integrin α and β surface expression patterns (Fig. 4A). Caki-1 markedly expressed α3 and β1, moderately expressed α5 and β3, whereas α1, α2, α4, α6 and β4 were only marginally detectable. KTC-26 strongly expressed α3 and β1. The subtype members α1, α2, α5, α6, β3 and β4 were moderately expressed, and α4 was not detectable. The integrin expression profile of A498 was similar to that of KTC-26, aside from α4, which was detectable for A498. We also noted that β4 was present in KTC-26 cells but not in A498 cells.

Amygdalin application for twenty-four hours and for 2 weeks altered the integrin surface profile, which is specific to the cell type (Fig. 4B). We noted that α5 and α6 were significantly down-regulated in all cell lines after 2 weeks of amygdalin exposure. β1, which was strongly expressed in all three cell lines, was also significantly reduced following 2 weeks of amygdalin application. The high basal expression of the α3 receptor was reduced in Caki-1 and KTC-26 but not in A498 by amygdalin. Differences were also noted in relation to α2 and β3, both of which were reduced in Caki-1 cells but elevated in KTC-26 and A498 cells after 2 weeks. Diminished expression levels of β4 were found in Caki-1 and KTC-26 cells after amygdalin exposure.

**Amygdalin influences the total cellular integrin content.** Evaluation of the integrin protein content after 24 h of amygdalin exposure revealed significant upregulation of α2 and downregulation of α3 and p-FAK (Fig. 5). β1 was significantly elevated in Caki-1 and KTC-26, whereas α6 was significantly decreased in Caki-1 and A498, and the total content of α5 was reduced in A498 cells after 24 h. In Caki-1 cells, α4 and β4 increased and β3 decreased.
After 2 weeks of exposure to amygdalin, integrin α2 significantly increased, even more so than after exposure for 24 h. In A498 cells, α3 and β3 increased after 2 weeks of amygdalin exposure. Reduced β1 and p-FAK occurred in KTC-26 cells, and β4 was downregulated in both Caki-1 and KTC-26 after 2 weeks of amygdalin exposure (Fig. 5).
Blocking experiments. The integrin expression profiles of all three cell lines were modified by amygdalin. Since surface integrin \( \alpha_5 \) and integrin \( \alpha_6 \) were strongly reduced in all three cell lines following amygdalin application, these integrins were chosen for functional blocking studies, to investigate whether the reductions correlate with changes in tumor cell adhesion and migration. Blocking \( \alpha_5 \) led to the significant inhibition of KTC-26 and A498 cell adhesion to collagen (Fig. 6A). However, adhesion of Caki-1 cells to collagen was not significantly influenced. Blocking integrin \( \alpha_6 \) receptor did not significantly affect adhesion to collagen in any of the cell lines (Fig. 6A) but significantly decreased chemotaxis in all three cell lines (Fig. 6B). Blocking the \( \alpha_6 \) receptor did not significantly affect adhesion to collagen in any of the cell lines (Fig. 6A) but significantly decreased chemotaxis in all three cell lines (Fig. 6B).

Discussion

It has been noted that interaction between tumor cells and endothelium plays a crucial role in metastatic progression; adhesion of non-small cell lung cancer (NSCLC) cells to the vessel-wall endothelium has been associated with tumor cell transmigration, which leads to brain and lymph node metastases (7). A more aggressive, metastasizing cancer phenotype has also been associated with enhanced adhesion (7). In the present study, we demonstrated that amygdalin exposure led to significant inhibition of the binding interaction between RCC cells and a HUVEC monolayer, collagen and fibronectin. The chemotactic and invasive activity of RCC cells was thereby inhibited. Such inhibition is clinically relevant since transendothelial migration and motile spreading are critical steps in tumor dissemination and progression (3) and correlate with poor survival. Reducing
migratory potential has been associated with successful tumor therapy and a less malignant tumor phenotype (8). Thus, we suggest that inhibiting RCC cell adhesion and motility by amygdalin reduces metastatic spread.

The adhesion- and migration-blocking effect of amygdalin is not restricted to RCC cells. Amygdalin has recently been demonstrated to also suppress the adhesive behavior of bladder cancer cells (5). Although amygdalin exerted a similar suppressive effect on the adhesion properties in bladder cancer and RCC cells, bladder cancer cell migration was affected differently by amygdalin. Chemotaxis was downregulated in two, but upregulated in one bladder cancer cell line following amygdalin exposure, indicating that the influence of amygdalin probably depends on the tumor entity. Thus, it is important to investigate the impact of amygdalin on different tumor entities.

The integrin family has been implicated in all steps of metastatic tumor progression (4,7). Integrin α5 is upregulated in tumor cells of epithelial origin, and a positive correlation between integrin α5 expression and RCC cell adhesion has been established (9). In the present investigation, amygdalin administration significantly decreased surface integrin α5 in all three cell lines. Also, the total cellular content of integrin α5 time-dependently decreased in the presence of amygdalin. Blocking integrin α5 function caused significant inhibition of KTC-26 and A498 cell adhesion to collagen and a decrease in the chemotactic activity of all cell lines employed. Consistent with the present data, downregulation of integrin α5 has previously been associated with reduced adhesive and invasive behavior of several cancer cell types (10-12).

In the present study, we noted that the collagen adhesion of Caki-1 cells did not decrease after blocking integrin α5, in contrast to the amygdalin-induced decrease in A498 and KTC-26 cells. Such a difference in integrin function between tumor cell types has previously been observed. Blocking α5 integrin has been shown to inhibit cell-matrix interaction of the bladder cancer cells HCV29 and BC3726 but enhance binding of the bladder cancer cell lines T24 and Hu456 (equipped with a different integrin set) (13). Similarly, blocking β1 integrin has been shown to inhibit UMUC-3 bladder cancer cell attachment to collagen, but has the opposite adhesive effect on TCCSUP cells, which are characterized by a different integrin expression profile (5). In the present investigation, amygdalin exposure caused surface β3 integrin to increase in KTC-26 and A498 cells, but decrease in Caki-1 cells. Counter-regulation involving another integrin subtype, in this case β3, may explain why blocking α5 failed to stop adhesion in Caki-1 cells. Loss of integrin α5 caused chemotactic reduction in all three cell lines. Therefore, we suggest that loss of surface integrin α5 is a mechanism by which amygdalin acts on RCC cell migration, and fine-tuning integrin performance depends on the specific integrin profile in the particular cell line.

Integrin α6 has been shown to facilitate epithelial cell migration and is correlated with progression risk, metastasis and death in clinical trials (14). Other studies have shown that integrin α6 promotes migration and invasion in colorectal cancer (15), and pancreatic (16) and breast (17) carcinomas. Integrin α6 has been noted to activate FAK (18) and FAK-related downstream signaling, which is relevant to controlling cell motility, survival and proliferation (4). In the present investigation, surface expression of integrin α6 was significantly reduced and FAK was deactivated by amygdalin in all three cell lines. Blocking surface integrin α6 demonstrated that α6 does not interfere with tumor cell adhesion but does regulate cell motility. Therefore, it is likely that reduction of α6 represents a mechanism by which amygdalin slows cell spreading.

Integrin β1 has been shown to promote cell invasion in breast, lung, pancreatic and colorectal cancer, as well as glioma, melanoma (4) and neuroblastoma (19). In prostate cancer cells, upregulation of integrin β1 has been shown to be accompanied by elevated motile behavior, whereas integrin β1 blockade contributes to the downregulation of chemotaxis, migration and cell adhesion (10). Inhibition of integrin β1 has been associated with reduced invasion and metastasis of ovarian cancer (12), and an integrin α5β1 peptide inhibitor has been shown to block breast cancer metastasis in vivo (20). In the present investigation, surface integrin β1 was downregulated in all three tumor cell lines following 2 weeks of amygdalin exposure. Therefore, amygdalin may act on integrin β1 to slow the motile spreading of RCC cells.

In vitro and in vivo investigations indicate that α3 is another integrin involved in the invasion of glioma, melanoma, hepatocellular and mammary carcinoma, and promotes lung metastasis of breast carcinoma cells (4). Blocking integrin α3 has resulted in adhesion inhibition of prostate cancer cells (21). In the present investigation, surface integrin α3 was reduced in Caki-1 and KTC-26, but not in A498 cells. This inhomogeneous reduction points to a cell line-specific effect induced by amygdalin.

Amygdalin application, besides modulating surface integrin expression, also changed total cellular integrin content. In the present study, total cellular integrin α2 expression was elevated in all three tumor cell lines following amygdalin exposure. Previous studies have shown that decreased levels of integrin α2 in tumor cells potentially increase tumor cell dissemination, and re-expression of integrin α2 has been shown to reverse malignant properties of breast cancer cells (22). Hence, we suggest that the amygdalin-induced inhibition of tumor cell adhesion and migration observed here is associated with upregulation of integrin α2.

Total cellular α3 integrin was decreased 24 h after amygdalin application in all three cell lines. This is in line with the amygdalin-induced reduction in surface α3 in Caki-1 and KTC-26 cells. Since surface α3 was not modified in the A498 cells, it is possible that amygdalin in this cell line acts through the intracellular α3 signaling pathways, p-FAK, which was strongly diminished in A498 cells 24 h after amygdalin application, supports this hypothesis since the α3-FAK axis is involved in cancer initiation and progression (23). Lee et al have demonstrated that FAK is a critical mediator of tumorigenesis and metastasis, which in part depend on integrin α3 (24). Therefore, we suggest that knocking down both integrin α3 and FAK deactivates the motile machinery of A498 cells.

Total cellular integrin β1 was elevated in Caki-1 cells after amygdalin application, while surface expression was diminished. This type of shift is not uncommon and points to receptor translocation from the surface to the intracellular compartment. Although the relevance of this process is not fully understood, integrin β1 trafficking to the plasma membrane has been shown to increase the metastatic potential of RCC cells, whereas stopping β1 recycling by maintaining
a high cytoplasmic and a low plasma membrane content decreases metastasis (25). Elevation of intracellular β1 has been shown to be linked with FAK deactivation (25), which correlates with the present findings.

The effects of amygdalin on integrin subtype expression depended on the cell line, upon whether the application time was acute (24 h) or chronic (2 weeks) and whether the integrin location was the cell surface or in the cytoplasm. The molecular mode of action of amygdalin in regard to integrin subtype expression, therefore, is not homogeneous and may influence both integrin-triggered mechanical cell-to-cell coupling and integrin-controlled biochemical pathway activation.

In the three investigated RCC cell lines, amygdalin application significantly reduced invasive and motile behavior. However, 2 weeks of amygdalin did not reduce the invasive capacity of KTC-26 cells. The reduction was predominantly associated with a decrease in surface α5 and α6 integrins. This mechanism, however, should not be generalized. Although amygdalin has been shown to inhibit adhesion and migration in bladder cancer cells as well, the integrins are altered differently. The α5 and α6 integrins seem to be an important target of amygdalin in RCC cells, whereas modulation of β1 or β4 integrins was most apparent in bladder cancer cells (5). Further in vitro investigations have been initiated to evaluate whether amygdalin also influences RCC cell growth, as has been observed for bladder cancer cells (6).

In conclusion, exposing RCC cells to amygdalin inhibits metastatic spread and is associated with downregulation of α5 and α6 integrins. Therefore, amygdalin exerts antitumor activity in vitro in RCC. This in vitro activity must be evaluated in an animal model.

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