Berberine, an isoquinoline alkaloid, inhibits melanoma cancer cell migration by reducing the expressions of cyclooxygenase-2, prostaglandin E\(_2\) and prostaglandin E\(_2\) receptors

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Malignant melanoma is the leading cause of death from skin disease due, in large part, to its propensity to metastasize. We have examined the effect of berberine, an isoquinoline alkaloid, on human melanoma cancer cell migration and the molecular mechanisms underlying these effects using melanoma cell lines, A375 and Hs294. Using an in vitro cell migration assay, we show that overexpression of cyclooxygenase (COX)-2, its metabolite prostaglandin E\(_2\) (PGE\(_2\)) and PGE\(_2\) receptors promote the migration of cells. We found that treatment of A375 and Hs294 cells with berberine resulted in concentration-dependent inhibition of migration of these cells, which was associated with a reduction in the levels of COX-2, PGE\(_2\) and PGE\(_2\) receptors (EP2 and EP4). Treatment of cells with celecoxib, a COX-2 inhibitor, or transient transfection of cells with COX-2 small interfering RNA, also inhibited cell migration. Treatment of the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), an inducer of COX-2 or PGE\(_2\), enhanced cell migration, whereas berberine inhibited TPA- or PGE\(_2\)-promoted cell migration. Berberine reduced the basal levels as well as PGE\(_2\)-stimulated expression levels of EP2 and EP4. Treatment of the cells with the EP4 agonist stimulated cell migration and berberine blocked EP4 agonist-induced cell migration activity. Moreover, berberine inhibited the activation of nuclear factor-kappa B (NF-kB), an upstream regulator of COX-2, in A375 cells, and treatment of cells with caffeic acid phenethyl ester, an inhibitor of NF-kB, inhibited cell migration. Together, these results indicate for the first time that berberine inhibits melanoma cell migration, an essential step in invasion and metastasis, by inhibition of COX-2, PGE\(_2\) and PGE\(_2\) receptors.

Introduction

The incidence of melanoma has increased dramatically in the past few decades in the USA (1,2) and is increasing rapidly in children (3). If recognized and treated early, melanoma is curable, but as the disease progresses, its propensity to metastasize makes it difficult to treat. The American Cancer Society estimated that in 2008, there were 8420 melanoma-associated deaths in the USA and the number of new cases of invasive melanoma was estimated at 62 480 (1,2). Ultraviolet (UV) radiation is a recognized risk factor for the development of skin cancers, including melanoma. Exposure of the skin to UV radiation induces an increase in the expression levels of cyclooxygenase (COX)-2, a rate-limiting enzyme that catalyzes the conversion of arachidonic acid to prostaglandins (PGs). Two COX isoforms with distinct physiologic functions have been identified. COX-1 is expressed constitutively in many tissues and has an important role in the maintenance of homeostasis. In contrast, COX-2 is an inducible enzyme that is activated by extracellular stimuli, such as UV radiation (4,5). The enhanced expression of COX-2 in skin exposed to UV radiation has been identified as a risk factor for the development of skin cancer (4,5). COX-2 generates PGs that are thought to play a central role in orchestrating the multiple events involved in cancer invasion and metastasis. PGE\(_2\) exerts its effects through its G-protein-coupled receptors—EP1, EP2, EP3 and EP4—and has been implicated in angiogenesis, decreased host immunity and enhanced invasion and metastasis (6,7). Since, melanoma is a highly malignant cancer with a potent capacity to metastasize distantly, an approach that decreases its metastatic ability or the ability of cancer cell migration may facilitate the development of an effective strategy for its treatment and/or prevention.

Berberine (Figure 1A), an isoquinoline alkaloid in nature, is found in the roots, rhizome and stem bark of a number of important medicinal plants, e.g., Berberis vulgaris (barberry), Berberis aquifolium (Oregon grape), Berberis aristata (tree turmeric) and Tinospora cordifolia. The potential effectiveness of berberine is indicated by its use in the Indian Ayurvedic (8), Unani and Chinese systems of medicine since time immemorial. Berberine possesses anticarcinogenic properties and appears to exhibit insignificant toxicity in normal human prostate epithelial cells and normal human epidermal keratinocytes (9,10). Berberine has been shown to inhibit inflammation in human hepatoma cells (11), effectively inhibit COX-2 transcriptional activity in human colon cancer cells (12,13) and inhibit DNA topoisomerase II (14). Therefore, we attempted to assess the effect of berberine on the migration potential of melanoma cancer cells using in vitro cell culture model, which has not been explored.

In this study, we assessed the chemotherapeutic effects of berberine on the migration of human melanoma cells, as the migration of tumor cells is a major event in the metastatic cascade. For this purpose, two melanoma cancer cell lines, A375 and Hs294, were selected, which exhibit metastatic characteristics. Normal human epidermal melanocytes were used as a control. In this study, we characterized the role of COX-2 and its metabolite PGE\(_2\) on the migration of human melanoma cancer cells and ascertained whether berberine has any suppressive effects on the COX-2-mediated migration of these cells. We also explored the involvement of various molecular targets in this process.

We present evidence that berberine inhibits melanoma cancer cell migration and that they do so through reducing the production of PGE\(_2\) by tumor cells in a process that involves the downregulation of the PGE\(_2\) receptors, EP2 and EP4, and the proteins of nuclear factor-kappa B (NF-kB) pathway using in vitro model.

Materials and methods

Cell lines and cell culture conditions

The human melanoma cell lines, A375 and Hs294, were purchased from the American Type Culture Collection (Manassas, VA). The cell lines were cultured as monolayers in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 100 \(\mu\)g/ml penicillin and 100 \(\mu\)g/ml streptomycin and maintained in an incubator with 5% CO\(_2\) at 37°C. The berberine was dissolved in a small amount of ethanol, which was added to the complete cell culture medium [maximum concentration of ethanol, 0.1% (vol/vol) in media] prior to addition to subconfluent cells (60–70% confluent). Cells treated with ethanol only served as a vehicle control. To determine the effect of berberine on 12-O-tetradecanoylphorbol-13-acetate (TPA)- or PGE\(_2\)-mediated effects, berberine was added in cell culture medium at least 30 min before the treatment of the cells with TPA, PGE\(_2\) or any other agent.
Berberine inhibits melanoma cell migration

The migration capacity of melanoma cancer cells with berberine for 24 h inhibits migration of cells in a concentration-dependent manner. COX-2 in A375 and Hs294 cells. Berberine inhibited melanoma cancer cell migration in vitro using Boyden Chambers (Neuroprobe, Inc., Gaithersburg, MD) in which the two chambers were separated with Millipore membranes (6.5 mm diameter filters, 8 µm pore size), as detailed previously (15). Briefly, melanoma chambers were assembled and kept in an incubator for 24 h. After 24 h, medium was replaced with phosphate-buffered saline and cells were photographed using an Olympus BX41 microscope and digital camera.

Chemicals and antibodies

Purified berberine was purchased from Sigma Chemical Co. (St Louis, MO). Antibodies specific for COX-2, an enzyme immunoassay kit for PGE2 analysis and an EP4 agonist (PGE1 alcohol) were obtained from Cayman Chemicals (Ann Arbor, MI). The antibodies specific for EP1, EP2, EP3 and EP4 and their secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). Celecoxib, PGE2 and TPA were purchased from Sigma Chemical Co. Boyden Chambers and polycarbonate membranes (8 µm pore size) for cell migration experiments were obtained from Neuroprobe (Gaithersburg, MD).

A375 cells were seeded in six-well plates and incubated overnight in starvation medium. Cells monolayers were wounded with a sterile 100 µl pipette tip, washed with starvation medium to remove detached cells from the plates. Cells were left either untreated or stimulated with the indicated doses of TPA and kept the cells for 24 h in an incubator. After 24 h, medium was replaced with phosphate-buffered saline and cells were photographed using an Olympus BX41 microscope and digital camera.

PGE2 immunoassay for quantitation of prostaglandin E2

The analysis of PGE2 in cell homogenates was performed using the Cayman PGE2 Enzyme Immunoassay Kit (Cayman Chemicals) following the manufacturer’s instructions. Briefly, at the indicated time points, cells were harvested and homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetracetic acid and 10 µM indomethacin using a homogenizer. Homogenates were centrifuged and the supernatants were collected and analyzed for PGE2 concentration according to the manufacturer’s instructions.

Berberine inhibits human melanoma cancer cell migration in a concentration-dependent manner

Molecular structure of berberine is shown in Figure 1A. We first determined whether treatment of A375 and Hs294 human melanoma cells with berberine inhibited their migration using Boyden chamber...
cells. Preliminary screening experiments were performed to determine the effects of lower concentrations of berberine (micrometers) that did not induce cell death. As shown in Figure 1B, relative to untreated control cells, treatment with berberine at concentrations of 0, 5, 10 and 20 μM reduced the migratory capacity of A375 and Hs294 cells in a concentration-dependent manner. The density of the migrating cells on the membrane after staining with crystal violet is shown in Figure 1B, and the numbers of migrating cells per microscopic field are summarized in Figure 1C. The cell migration was inhibited by 17–69% (P < 0.01–0.001) in A375 cells and by 27–74% (P < 0.01–0.001) in Hs294 cells in a concentration-dependent manner after treatment with berberine for 24 h. A similar but comparatively higher inhibitory effect was observed at the 48 h time point.

To confirm that the inhibition of cancer cell migration by berberine was a direct effect on migration and not due a reduction in cell viability, a trypan blue assay was performed using cells that were treated identically to those used in the migration assays. Treatment of A375 and Hs294 cells with various concentrations of berberine (0, 5, 10 and 20 μM) for 24 h had no significant effect on cell viability, change in cell morphology and cell death (data not shown). Similar experiments were also conducted with normal human epidermal melanocytes (Life Sciences, Carlsbad, CA). Under identical in vitro conditions, cell migration was not observed in normal human epidermal melanocytes (data not shown).

The inhibitory effect of berberine on cell migration is associated with the inhibition of endogenous COX-2 expression and PGE2 production.

To determine whether the inhibitory effect of berberine on the migration of the cells is associated with inhibition of basal COX-2 expression, we determined the levels of COX-2 in lysates of cells from the various treatment groups using western blot analysis. As shown in Figure 1D, treatment of A375 and Hs294 cells with berberine reduced the levels of COX-2 expression in a concentration-dependent manner as compared with the expression in untreated controls. As the COX-2 metabolite, PGE2, has been implicated in COX-2-mediated effects; we determined the levels of PGE2 in the berberine-treated cells. Our results revealed that treatment with berberine for 24 h resulted in significant inhibition of PGE2 production in both A375 (15–75%, P < 0.01–0.001) and Hs294 (16–63%, P < 0.01–0.001) cells in a dose-dependent manner (Figure 2A), suggesting that berberine-induced reduction in PGE2 production is associated with an inhibitory effect of the berberine on COX-2 in these cells.

Celcoxib, a COX-2 inhibitor, inhibits melanoma cell migration.

This experiment was performed to determine whether the inhibitory effect of berberine on melanoma cell migration is mediated through its inhibitory effect on COX-2 expression. For this purpose, equal numbers of A375 and Hs294 cells were subjected to the cell migration assay after treatment with various concentrations of celcoxib (0, 5, 10, 20 μM) for 24 h. As shown in Figure 2B, treatment of the cells with celcoxib resulted in a dose-dependent reduction in the cell migration capacity of melanoma cells as compared with non-celcoxib-treated controls (P < 0.01–0.001). These data suggested that the inhibition of constitutive levels of COX-2 expression in the presence of celcoxib resulted in inhibition of melanoma cancer cell migration.

siRNA knockdown of COX-2 leads to reduction of melanoma cell migration.

We further verified the role of COX-2 in cell migration through siRNA knockdown of COX-2 in the melanoma cells and examined whether it would lead to the inhibition of the cell migration in these cells. The transfection of A375 and Hs294 cells with COX-2 siRNA resulted in significant reduction of cell migration in A375 (85%, P < 0.001) and Hs294 (86%, P < 0.001) cells after 24 h as compared with the migration of control siRNA-transfected A375 and Hs294 cells (Figure 2C). We also confirmed using western blot analysis that COX-2 siRNA transfection of A375 and Hs294 cells resulted in marked reduction in the levels of COX-2 protein (>80%) in these cells (Figure 2D). As the inhibitory effect of berberine on the migration of A375 and Hs294 cell lines was very similar, the subsequent studies were performed using A375 cells.

Berberine inhibits TPA-induced cell migration of melanoma cells.

Treatment of skin with TPA stimulates the levels of COX-2 expression (17,18); therefore, we determined the effects of TPA on the melanoma cell migration. As shown in Figure 3A, treatment of A375 cells with TPA for 24 h resulted in significantly enhanced cell migration (P < 0.01, P < 0.001). The inhibitory effect of berberine versus non-berberine-treated controls, **P < 0.01**. (B) Downregulation of endogenous COX-2 reduces cancer cell migration. Treatment of A375 and Hs294 cells with celcoxib, a COX-2 inhibitor, inhibits cell migration in a dose-dependent manner. The data are expressed as the mean number of migratory cells ± SD per microscopic field. Significant difference versus control (non-celecoxib-treated) cells, *P < 0.01, **P < 0.001*. (C) Transfection of cells, both A375 and Hs294, with COX-2 siRNA significantly decreases cell migration. A375 and Hs294 cells were transfected with COX-2 siRNA to knockdown COX-2 expression. Significant reduction of cell migration versus control siRNA-treated cells, **P < 0.001**. (D) Transfection of A375 and Hs294 cells by COX-2 siRNA resulted in marked reduction in the levels of COX-2 protein in cells.
Berberine inhibits melanoma cell migration

Next, we examined whether berberine inhibits PGE2-induced cell migration in human melanoma cells. For this purpose, A375 cells were treated with various concentrations of PGE2 (0, 5, 10 and 20 μM) for 24 h and their migration determined. In another set of experiments, a cell migration assay was performed in which A375 cells were treated with berberine (10 and 20 μM) for 24 h resulted in inhibition of TPA-induced COX-2 expression (Figure 3E). These results suggest that berberine inhibits TPA-induced cell migration through the downregulation of COX-2 expression.

Berberine inhibits PGE2-induced cell migration of melanoma cells

As it has been shown that PGE2 manifests its biological activity via four known G-protein-coupled receptors (i.e. EP1–EP4) (7,19), we determined the effect of berberine on the basal levels of PGE2 receptors in melanoma cells. Western blot analysis revealed that treatment of A375 cells with berberine (0, 5, 10 and 20 μM) for 24 h resulted in a dose-dependent reduction in the levels of EP2 and EP4 (Figure 5A). A similar inhibitory effect on EP1 and EP3 was observed but was less prominent than the effect on EP2 and EP4 (data not shown).

In further experiments, we tested whether berberine inhibits the PGE2 induction of higher levels of the EP2 and EP4 receptors in melanoma cells. For this purpose, the A375 cells were treated with PGE2 (10 μM) with and without the treatment of berberine (10 and 20 μM) for 24 h, then cells were harvested and cell lysates prepared. As shown in Figure 5B, western blot analysis indicated that the treatment of cells with PGE2 enhanced the levels of both EP2 and EP4 and that berberine inhibited the PGE2-induced increase in the levels of EP2 and EP4.

An EP4 agonist enhances the cell migration of melanoma cells and berberine inhibits EP4 agonist-induced cell migration

To assess the role of the PGE2 receptors on the cell migration of melanoma cells and the therapeutic effect of berberine, A375 and Hs294 melanoma cells were treated with an EP4 agonist (PGE1 alcohol) for 24 h with or without addition of berberine. As shown in Figure 5C, treatment of A375 and Hs294 cells with the EP4 agonist resulted in significant enhancement of cellular migration (76 and 95%,
a treatment of berberine resulted in the downregulation of IKK concentration-dependent manner (Figure 6B). The results also indicated and degradation of IκB in a dose-dependent manner (Figure 6A). The activity of NF-κB is an important mediator of melanoma cell migration (18–71%) relative to untreated control cells and with caffeic acid phenethyl ester resulted in a dose-dependent reduction of cell migration (18–71%) relative to untreated control cells and with caffeic acid phenethyl ester (CAPE), an inhibitor of NF-κB, for 24 h inhibits cell migration in a concentration-dependent manner. Data on cell migration capacity are summarized as the mean number of migratory cells ± SD per microscopic field. Significant inhibition versus non-CAPE-treated cells: **P < 0.001.

Berberine decreases the basal level and activity of NF-κB/p65 in melanoma cells: NF-κB is an important mediator of melanoma cell migration

COX-2 is a downstream target gene of NF-κB, therefore we assessed whether berberine also affects the basal levels of proteins of NF-κB family in melanoma cells. For this purpose, A375 cells were treated with various concentrations of berberine (0, 5, 10 and 20 µM) for 24 h, and thereafter cells were harvested and cell lysates prepared. The results of western blot analysis revealed that treatment of cells with berberine downregulated the translocation of NF-κB/p65 from the cytosol to the nucleus in a dose-dependent manner (Figure 6A). The activity of NF-κB also was significantly reduced (15–80%, P < 0.01 and P < 0.001) after the treatment of cells with berberine in a concentration-dependent manner (Figure 6B). The results also indicated that treatment of berberine resulted in the downregulation of IKKα and degradation of IκBα (Figure 6A), which leads to the inactivation of NF-κB and its translocation to the nucleus. To check whether NF-κB has a role in melanoma cell migration, A375 melanoma cells were treated with various concentration of caffeic acid phenethyl ester (0, 5.0, 10.0 and 20.0 µg/ml), a potent inhibitor of NF-κB, and cell migration was determined. As shown in Figure 6C, treatment of cells with caffeic acid phenethyl ester resulted in a dose-dependent reduction of cell migration (18–71%) relative to untreated control cells and similar to that observed on treatment of the cells with berberine (Figure 1A).

Discussion

Melanoma is the leading cause of death from skin diseases and can metastasize very rapidly. Although less common than other types of skin cancer, it causes the majority (75%) of skin cancer-related deaths. According to a World Health Organization report, 48 000 melanoma-related deaths occur worldwide per year (20). Treatment is more difficult if it has spread beyond skin and lymph nodes (21). Once diagnosed with metastatic melanoma, most patients will ultimately die of their disease within 2 years (22). Therefore, innovative strategies are required to be developed for the prevention of the invasive or the migratory potential of melanoma cells. Many human cancers express elevated levels of COX-2, an enzyme responsible for the biosynthesis of PGs. COX-2 overexpression and abundant production of
PGs, and particularly PGE$_2$, have been linked with tumor progression, invasion and metastasis (23). Because of its important role in tumor invasion and metastasis, COX-2 is not a new target but a promising target for cancer therapy (7,24); therefore, the search of potential COX-2 as well as PGE$_2$ inhibitors for the prevention or treatment of melanoma may prove to be an important strategy.

The significant findings in the present study are that the treatment of melanoma cells with berberine for 24 h inhibits cell migration in a dose-dependent manner and that is associated with the inhibition of COX-2 expression and PGE$_2$ production. Based on our observation, cells will go under apoptosis or cell death if melanoma cells are treated with berberine for $>24$ h time period. Under these conditions, cell migration will decrease, and this reduction in cell migration could be due to cell death and not because of changes in migrating behavior of cells. In our study, cell death or apoptosis is not a reason of berberine-caused inhibition of melanoma cell migration. The melanoma cells overexpress COX-2, and the inhibition of COX-2 by berberine may contribute to the inhibition of cell migration of these cells. This concept is supported by the evidence that treatment of the melanoma cells with celecoxib, a potent COX-2 inhibitor, resulted in a reduction in cell migration. Similar effects were also noted when the melanoma cancer cells, A375 and Hs294, were transfected with COX-2 siRNA. It has been shown that TPA activates COX-2 expression, and we found that treatment of melanoma cells with TPA enhances cell migration and that this TPA-induced cell migration was blocked by the treatment of cells with berberine. These observations support the evidence that inhibition of melanoma cell migration by berberine requires the inhibition of COX-2 expression.

It is well known that PGE$_2$ exerts its biologic functions through four G-protein-coupled receptors, EP1, EP2, EP3 and EP4 (7,19,25), that can stimulate epithelial cell growth, invasion potential and cellular survival signals (26–28). It has been shown very recently that PGE$_2$ promotes lung cancer cell migration and that this effect is mediated through activation of PGE$_2$ receptors (29). Because PGE$_2$ is a major prostaglandin associated with skin tumor promotion, progression and invasion (23), we assessed the possible involvement of the PGE$_2$ receptors in berberine-induced inhibition of melanoma cell migration. We observed that A375 and Hs294 cells overexpress the PGE$_2$ receptors, EP2 and EP4, and that the expression of EP2 and EP4 was decreased when cells were treated with berberine in vitro. These data suggest that inhibition of EP2 and EP4 levels by berberine may contribute to the inhibition of cell migration. The inhibitory effect of berberine on melanoma cancer cell migration through the inhibitory effect on EP2 or EP4 was further confirmed by treating the cells with an EP4 agonist. We found that the treatment of A375 and Hs294 cells with the EP4 agonist resulted in enhancement of cell migration, and that EP4 agonist-induced cell migration was inhibited by the treatment of cells with berberine. This observation supports the concept that inhibition of PGE$_2$ receptors by berberine may have contributed to the inhibition of melanoma cancer cell migration. These findings also demonstrate the feasibility of using berberine as an alternative to COX-2 inhibitors, which show toxicity in some patients, given the fact that COX-2 remains an attractive cancer target. Since berberine acts by inhibiting expression of both COX-2 and EP receptors, this could be more effective since it targets both ligand (PGE$_2$) and receptor (EP). Similar to berberine, other phytochemicals also have been examined for their effect on cell migration. Punathil et al. (30) have reported that treatment of non-small-cell lung cancer cells with proanthocyanidins resulted in inhibition of cell migration following the inhibition of nitric oxide and guanylate cyclase pathways. Another dietary polyphenol, (-)-epigallocatechin-3-gallate from green tea, has been shown to inhibit mammary cancer cell migration through the inhibition of nitric oxide and nitric oxide-mediated mechanisms (15).

As COX-2 is a downstream target of NF-kB pathway, we further checked the effect of berberine on the basal levels of NF-kB in melanoma cells and found that treatment of melanoma cells with berberine downregulated the basal levels of proteins of NF-kB pathway in a dose-dependent manner. Treatment of melanoma cells with caffeic acid phenethyl ester, an inhibitor of NF-kB, resulted in an inhibitory effect on cell migration. These observations further support the concept that the inhibitory effect of berberine on melanoma cell migration is mediated, at least in part, through the downregulation of COX-2, PGE$_2$ and PGE$_2$ receptors expressions. However, it remains possible that downregulation of other NF-kB target genes could also contribute to the inhibition of cell migration.

In summary, the results from this study have identified for the first time that berberine inhibits the migration of melanoma cancer cells through the inhibitory effect on endogenous COX-2 overexpression and successive downregulation of PGE$_2$ and PGE$_2$ receptors. More mechanism-based studies are therefore needed to develop berberine as a pharmacologically safe agent alone, or in combination with other anti-metastatic drugs, for the treatment of metastatic melanoma in humans.

Funding
Veterans Administration Merit Review Award to S.K.K.

Conflict of interest statement: None declared

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Received August 29, 2010; revised September 28, 2010; 
accepted October 15, 2010