Aspirin Suppresses the Growth and Metastasis of Osteosarcoma through the NF-κB Pathway

Dan Liao, Li Zhong, Tingmei Duan, Ru-Hua Zhang, Xin Wang, Gang Wang, Kaishun Hu, Xiaobin Lv, and Tiebang Kang

Abstract

Purpose: Aspirin has recently been reported to reduce both the incidence and the risk of metastasis in colon cancer. However, there is no evidence at the cellular levels or in the animal models for such an effect of aspirin on cancer metastasis.

Experimental Design: MTT assay, colony formation assay, and apoptosis assay were employed to analyze the effects of aspirin on the osteosarcoma cell viability in vitro. The NF-κB activity was measured by the NF-κB p65 luciferase reporter. Western blotting was used to analyze the proteins in cells. The migration and invasion abilities of osteosarcoma cells in vitro were measured by the Transwell assay. Xenograft-bearing mice were used to assess the roles of aspirin in both tumor growth and metastasis of osteosarcoma in vivo (n = 5–8 mice/group). An unpaired Student t test or ANOVA with the Bonferroni post hoc test were used for the statistical comparisons.

Results: Aspirin reduced cell viability in a dose- and time-dependent manner in osteosarcoma cell lines, and aspirin synergistically sensitized osteosarcoma cells to cisplatin (DDP) in vitro and in vivo (P < 0.001). Moreover, aspirin markedly repressed the migration and invasion of osteosarcoma cells in vitro (P < 0.001), and dramatically diminished the occurrence of osteosarcoma xenograft metastases to the lungs in vivo (P < 0.001). Mechanistically, aspirin diminishes osteosarcoma migration, invasion, and metastasis through the NF-κB pathway.

Conclusion: Aspirin suppresses both the growth and metastasis of osteosarcoma through the NF-κB pathway at the cellular level and in the animal models to argue that aspirin may be an excellent auxiliary drug for treating patients with osteosarcoma.

Materials and Methods

Reagents and cell culture

U2OS and MG63 cells were cultured according to the instructions from the ATCC. ZOS and U2OS/MTX300 cells were described previously (15). HEK-293T cells were purchased from the ATCC, cultured in DMEM (Invitrogen) with 10% FBS ( Gibco), 1 mmol/L glutamine, and 100 U/ml each of penicillin and streptomycin. All cell lines used in this study were authenticated using short-tandem repeat profiling less than 6 months ago when this project was initiated, and the cells have not been in culture for more than 2 months.

Aspirin was purchased from Sigma-Aldrich. Antibodies against p65(C22B4), XIAP(3B6), CIAP1(D5G9), CIAP2(58C7), BCL2(50E3), Survivin(6E4), Livin(D61D1), and α-Tublin(2148S) were purchased from Cell Signaling Technology. An antibody against GAPDH(SC-166574) was purchased from Santa Cruz Biotechnology.

Plasmids

The full-length p65 cDNA was cloned into the pBABE vector from human 293T cells. P65 was generated by PCR amplification, subcloned into the pBABE and pCDNA3.1 vectors, and verified by DNA sequencing. The p65 shRNA was purchased from Sigma-Aldrich. The CMV-luciferase plasmid was a gift from Prof. Xiaoming Xie (Sun Yat-Sen University, Guangzhou, China; ref. 16).

Cell viability assay

U2OS, MG63, or ZOS cells were seeded in 96-well plates at a density of 3,000 cells per well. They were treated with different...
concentrations of aspirin (0, 1, 2.5, 5, 10, or 20 mmol/L) and/or chemotherapy drugs (DDP, 10 μmol/L) for the indicated times, and the cell viability was measured by MTT assays as described previously (15, 17).

Clone formation assay

Colony formation assays were performed as described previously (15, 18). ZOS or U2OS cells plated in triplicate at 500 cells per well in 6-well plates were treated with vehicle (DMSO), 10 μmol/L DDP, 5 mmol/L aspirin, or 10 μmol/L DDP plus 5 mmol/L aspirin for 48 hours, and then cultured for 10 days. The cell clones were washed with PBS, and then fixed in methanol and dyed with 0.1% crystal violet, and the colonies that contained more than 50 cells were counted.

Apoptosis flow-cytometry assay

Drug-induced apoptosis was detected using an Annexin V-EGFP apoptosis detection kit (KeyGEN). Treated ZOS, MG63, or U2OS cells [negative control, 10 μmol/L DDP, 5 mmol/L aspirin, or 10 μmol/L DDP plus 5 mmol/L aspirin for 48 hours] were harvested, the assay were performed according to the manufacturer’s instructions. The stained cells were analyzed using a Cytomics FC500 flow cytometer (Beckman Coulter).

The effect of combination of DDP and aspirin

This effect of the combination of DDP and aspirin was assessed by the Chou-Talalay method (19, 20). Briefly, the cells were treated with serial dilutions of DDP and aspirin separately or simultaneously for 48 hours, and the viability of the cells was quantified using MTT assays. A combination index (CI) was calculated using CalcuSyn software following the equation: CI = (D1)(D2)((D1)(D2))1/((D1)(D2)), where CI = 1.0, < 1.0, and > 1.0, respectively.

Transfection experiments

p65 siRNAs were synthesized by GenePharma. Transfections were performed according to the manufacturer’s instructions using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) and 50 nmol/L siRNA. The transfections were performed as described previously (15). Briefly, asynchronously growing cells seeded at 2.5 × 10^5 cells per well in a 6-well plate or at 1 × 10^6 cells in a 10-cm plate were transfected with 2 or 12 μg plasmid DNA, respectively, using Lipofectamine 2000 (Invitrogen). Cell lines that stably expressed the NC or p65 shRNAs were established by the Sigma shRNA system according to the manufacturer’s instructions.

Dual-luciferase reporter assay

Transfection was performed as described previously (15). The transfected p65-siRNA cells incubated for 24 hours or U2OS cells stably overexpressing p65 were transfected with NF-kB p65 luciferase reporter and pRL-TK Renilla luciferase construct (Promega) per well using Lipofectamine 2000 (Invitrogen). After 24 hours, the cells were treated with aspirin (5 mmol/L). The cells were then analyzed after an additional 24 hours according to the Dual-Luciferase Assay System protocol (Promega).

Boyden chamber assays

The migration and invasion of osteosarcoma cells were examined using 24-well Boyden chambers with 8-μm inserts coated without (migration) or with Matrigel (invasion) as previously described (21). A total of 5 × 10^5 cells per well were plated on the inserts and cultured at 37°C in the upper chambers without serum and supplemented with Aspirin (5 mmol/L). After 24 hours, the cells that crossed the inserts were stained with crystal violet (0.005%, sigma) and counted as the number of cells per field of view under phase-contrast microscopy.

Western blotting

Western blotting was performed as previously described (18). Briefly, the cells were lysed in RIPA buffer containing protease inhibitor and phosphatase inhibitor cocktails (Thermo Scientific). The nuclear protein was isolated according to the protocol provided by the Nuclear Protein Extraction Kit (Thermo Scientific). The protein extracts were resolved using SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blotting using ECL detection reagents (Beyotime Co. Haimen).

Mouse xenograft

Animal experiments were approved by the Animal Research Committee of Sun Yat-sen University Cancer Center and were performed in accordance with established guidelines. For osteosarcoma xenograft growth of orthotopic animal model, U2OS/MTX300 cells were used as previously described (15, 22). Two weeks after the cells were injected, the mice were randomly separated into four groups (n = 6). The mice were treated with DMSO, aspirin (100 mg/kg) by intragastric administration every day, and/or DDP (3 mg/kg) by i.p. injection every week. The mice were monitored every 2 days for tumor formation and sacrificed when the tumors reached approximately 1.5 cm in diameter, and the tumor xenografts were harvested and weighted. The tumor volume was calculated using the formula V = 4/3 πr^2h, as described previously (22).

For the spontaneous metastasis model, human osteosarcoma cells were transplanted orthotopically into the bones of mice as previously described (15, 22). We used 143B cells stably expressing luciferase (143B-luci) or U2OS/MITX300 cells stably expressing luciferase (U2OS/MITX300-luci) and U2OS/MITX300-luci-derived cells, including U2OS/MITX300-p65sh#1, -p65sh#2, -vector and -p65-bearing stable knockdowns of p65,
Aspirin inhibits the proliferation and promotes apoptosis of osteosarcoma cells. A, aspirin inhibits osteosarcoma cell (U2OS, MG63, and ZOS) viability in a time- and dose-dependent manner. Osteosarcoma cells were treated with different concentrations of aspirin for the indicated times, and the viability of these cells was measured using MTT assays; \( P < 0.01 \). A two-sided ANOVA with a Bonferroni post hoc test was used for statistical analysis. B and C, aspirin augments the chemosensitivity of osteosarcoma cells to DDP. B, the indicated cells were treated with 10 \( \mu \)mol/L DDP with or without 7.5 mmol/L aspirin for 48 hours as indicated, and the viability of these cells was measured by MTT assays; \( n = 3 \); \( P < 0.01 \). (Continued on the following page.)
overexpressing vector, and p65, respectively. One week after the cells were injected, the mice were randomly separated into two groups \((n = 6)\). The mice were treated with DMSO or aspirin \((100 \text{ mg/kg})\) by intragastric administration every day. When the xenografts reached 1.5 cm in diameter, the lungs of mice bearing the osteosarcoma tumor xenografts that stably expressed luciferase were analyzed using an IVIS Lumina Imaging System (Xenogen). The mice were then sacrificed, the lungs of the mice were weighed, and the number of metastatic nodules in the lungs was counted. The harvested lungs were fixed in formalin \((4 \text{ mol/L})\) and stained with hematoxylin and eosin \((\text{H&E})\) for histologic assessment, and total RNA was extracted for quantitative real-time reverse transcription-PCR \((\text{qRT-PCR})\) analysis of human HPRT mRNA expression.

**qRT-PCR**

qRT-PCR was performed using a LightCycler 480 instrument (Roche Diagnostics) and SYBR Premix Ex Taq \((\text{TaKaRa})\) according to the manufacturer's instructions. All reactions were carried out in a 10 \(\mu\text{L}\) reaction volume in triplicate. The primers for GAPDH were obtained from Invitrogen. Standard curves were generated, and the relative amount of target gene mRNA was normalized to that of GAPDH. The specificity was verified by melting curve analysis. To quantify cancer metastasis in mouse lungs, qRT-PCR for human hypoxanthine-guanine-phosphoribosyl transferase \((\text{hHPRT})\) was performed on TRIzol \((\text{Invitrogen})\)-isolated total RNA using primers for hHPRT and 18S rRNA. The oligonucleotide sequences of the qRT-PCR primers are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>primer (F: forward, R: reverse)</th>
</tr>
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<tbody>
<tr>
<td>P65 F:</td>
<td>5’-AGCTCAAGATCTGCGAGTG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-ACATGACGTGACAAAGGA-3’</td>
</tr>
<tr>
<td>GAPDH F:</td>
<td>5’-ATCCACATCCAGGAGCA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CTTCTCCATGGTGGTGAAGAC-3’</td>
</tr>
<tr>
<td>hPRT F:</td>
<td>5’-TCTCTTGTCAAGCGATATACTCC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-AGTCTGCTATATCACAACCTC-3’</td>
</tr>
<tr>
<td>18sRNA F:</td>
<td>5’-CGGCCACATCAACAGAAGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCTGGAATTACCGCCGCT-3’</td>
</tr>
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**Statistical analysis**

The data are presented as the mean \(\pm\) SD. The error bars indicate the SD. Two-sided ANOVA with the Bonferroni post hoc test was used to compare the differences between subgroups \(*, P < 0.05\); **, \(P < 0.01\) and ***, \(P < 0.001\) was considered to be strongly significant.
Results

Aspirin inhibits the osteosarcoma growth in vitro and in vivo

We recently showed that the inhibition of the NF-κB pathway impeded the growth of osteosarcoma and increased the sensitivity of osteosarcoma to chemotherapies in vitro and in vivo (15). It is well known that aspirin inhibits the activity of NF-κB (23, 24) and the aspirin induces apoptosis in many cancers (25–28), we reasoned that aspirin would improve the efficiency of osteosarcoma to chemotherapies. Using the osteosarcoma cell lines, including U2OS, ZOS, and MG63, we observed that aspirin reduced cell viability in a dose- and time-dependent manner (Fig. 1A). Because patients with osteosarcoma often develop chemoresistence in clinic, we sought to determine whether aspirin sensitizes osteosarcoma cells to DDP, the common used chemotherapeutic drug in osteosarcoma patients. As shown in Fig. 1B and C, Supplementary Fig. S1, the combination of DDP with aspirin was more efficiently decreasing the osteosarcoma cell viability and the clone formation ability (P < 0.05). Furthermore, Aspirin sensitized osteosarcoma cells to the DDP-induced apoptosis in vitro (Fig. 1D; Supplementary Fig. S2). In addition, the combination of DDP with aspirin was synergistic at low concentrations showed by Fa-CI plots (Fig. 1E). Collectively, these results indicate that aspirin inhibits the proliferation of osteosarcoma cells and promotes the apoptosis of osteosarcoma cells induced by DDP in vitro.

Next, we investigated whether aspirin could inhibit the osteosarcoma growth using the osteosarcoma orthotropic animal model in vivo. As shown in Fig. 2B, sacrificed the tumor-bearing mice at the 24th day, we found the combination of aspirin and DDP showed more significant inhibition of tumor growth, compared with either aspirin or DDP alone (P < 0.001). As shown in Fig. 2A and C, consistent with the tumor growth curve, the tumor weights and volumes were more decreased in the group of combining aspirin with DDP, compared with aspirin or DDP
alone (P < 0.01). More importantly, the combination of aspirin and DDP did not increase their toxicities in vivo, as indicated by mouse body weight (Fig. 2D). Taken together, these results reveal that aspirin enhances the sensitivity of osteosarcoma to chemotherapies in vitro and in vivo.

**Aspirin inhibits the osteosarcoma metastasis in vivo**

Aspirin has recently been reported to reduce both the incidence and the risk of metastasis in colon cancer (7), and osteosarcoma is often resistant to the conventional chemotherapy drugs because of high rate of lung metastasis (29), we sought to determine whether aspirin has inhibition on osteosarcoma metastasis in vivo. As shown in Fig. 3A; Supplementary Fig. S3, aspirin markedly suppressed the migration and invasion of osteosarcoma cells as measured by Boyden chamber assays in vitro (P < 0.01). More strikingly, using a spontaneous in vivo metastasis model of human osteosarcoma cells (Supplementary Fig. S4), and evaluated cancer metastasis to the lungs when xenografts reached 1.5 cm in diameter. Luminescence imaging for the lungs identified the severity of lung metastasis was inhibited by the aspirin (Fig. 3B). Aspirin dramatically diminished the occurrence of osteosarcoma xenograft metastases to the lungs, as indicated by the number of metastatic nodules in the lung (Fig. 3C, P < 0.05) and the wet lung weight (Fig. 3E, P < 0.01). H&E staining also showed that aspirin reduced the metastasis in the lungs of the mice bearing U2OS/MITX300 or 143B xenografts compared with DMSO treatment (Fig. 3D). In addition, the human HPRT mRNA levels detected by real-time PCR were decreased by 89% in U2OS/
Figure 5.
Aspirin represses the osteosarcoma migration and invasion via the NF-κB pathway in vitro. A and B, aspirin has a minimal effect on the NF-κB transcriptional activity in both U2OS and ZOS cells knockdown of p65. The indicated cells were transfected with negative control or p65-siRNA for 24 hours and then transfected with an NF-κB luciferase reporter for 24 hours. The cells were treated with or without aspirin (5 mmol/L) for 24 hours, and then subjected to luciferase assays (B) as described in Materials and Methods. The efficiency of p65-siRNA was determined by Western blotting (A); n = 3. Bars, SD; **, P < 0.01. A two-sided ANOVA with a Bonferroni post hoc test was used for statistical analysis. C, aspirin has no effect on the invasion and migration abilities of osteosarcoma cells knockdown of p65. The indicated cells transfected with or without p65-siRNA (as indicated) for 24 hours were treated with or without aspirin (5 mmol/L) for 24 hours, and then the migration and invasion assays (as indicated) were subsequently performed as described in Materials and Methods; n = 3; **, P < 0.01. A two-tailed Student t test was used for statistical analysis. D and E, the effect of aspirin on the NF-κB transcriptional activity is rescued in both U2OS and ZOS cells stably overexpressing p65. The indicated stable transfectants (D) were transfected with an NF-κB luciferase reporter for 24 hours. The cells were treated with or without aspirin (5 mmol/L) for 24 hours, and then subjected to luciferase assays (E) as described in Materials and Methods; n = 3. Bars, SD; **, P < 0.01. A two-sided ANOVA with a Bonferroni post hoc test was used for statistical analysis. F, the impairment of aspirin on migration and invasion are rescued in both U2OS and ZOS cells stably overexpressing p65. The indicated stable transfectants were treated with or without aspirin (5 mmol/L) for 24 hours, and then subjected to migration and invasion assays as described in Materials and Methods; n = 3. Bars, SD; **, P < 0.01. A two-sided ANOVA with Bonferroni post hoc test was used for statistical analysis.
MTX300 xenografts and 77% in 143B xenografts model, respectively (Fig. 3E). Collectively, these results demonstrate that aspirin inhibits osteosarcoma metastasis in vivo.

Aspirin inhibits osteosarcoma metastasis through the NF-κB pathway

Aspirin has been shown to inhibit the activity of NF-κB, which plays key roles in diverse physiologic and pathologic processes, including apoptosis, proliferation, migration, invasion, and metastasis (30–32). In addition, we recently showed that the inhibition of the NF-κB pathway impaired the growth of osteosarcoma and increased the sensitivity of osteosarcoma to chemotherapies in vitro and in vivo (15). Therefore, we were very curious to determine whether aspirin also had an inhibitory effect on the osteosarcoma metastasis by modulating the NF-κB pathway. Osteosarcoma cells transfected with NF-κB luciferase plasmid, and then incubated with aspirin for 24 hours. The p65 NF-κB luciferase reporter activity was decreased by 50% after treated with aspirin in osteosarcoma cells (Fig. 4A, \( P < 0.01 \)). Consistently, the nuclear localization of p65 protein, the indicator of NF-κB transcription activity, was also decreased in osteosarcoma cells treated with aspirin (Fig. 4B). Consequently, the NF-κB–targeted genes, such as CIAP, XIAP, BCL2, and Survivin, were inhibited by aspirin in a dose- and time-dependent manner in both ZOS and U2OS cells (Fig. 4C).

Next, we explored whether the inhibition of aspirin on osteosarcoma metastasis depends on the NF-κB pathway. First, as shown in Fig. 5A–C; Supplementary Fig. S5, aspirin had no effect...
on the NF-κB luciferase reporter activity, the migration and invasion of osteosarcoma cells knockdown of p65 by siRNA in these cells (P > 0.05). Conversely, as shown in Fig. 5D–F, Supplementary Fig. S6, the impairment of aspirin on these events were completely rescued by in these osteosarcoma cells overexpressing p65. Second, we stably knocked down and overexpressed the p65 in U2OS/MTX300-luci cells, which were identified by Western blotting (Fig. 6A). Using a spontaneous metastasis model in vivo and the assays mentioned above, as shown in Fig. 6B–E, the inhibition of osteosarcoma metastasis to the lungs by aspirin was not further enhanced in the cells bearing a stable knockdown of p65 by shRNA, but was abolished in the cells stably overexpressing p65. These results demonstrate that Aspirin diminishes both the growth and metastasis of osteosarcoma through the NF-κB pathway.

**Discussion**

Patients with metastatic or relapsed osteosarcoma have a poor prognosis and new therapies are needed. Aspirin has recently been reported to reduce the incidence and risk of metastasis in colon cancer patients (7). In the present study, we demonstrated that aspirin diminishes the growth and metastasis of osteosarcoma through the NF-κB pathway in vitro and in vivo.

Aspirin can inhibit the activity of NF-κB (23, 24), and the inhibition of the NF-κB pathway repressed the growth of osteosarcoma and increased the sensitivity of osteosarcoma to chemotherapies in vitro and in vivo (15). Thus, it was reasonable to speculate that aspirin may sensitize osteosarcoma cells to DDP. Indeed, this was the case, as our results indicated that the combination of DDP and aspirin was more efficient in killing osteosarcoma cells in vitro and in vivo than either DDP or aspirin alone. This is consistent with the fact that regular and long-time intake of aspirin with a significant reduction in the prevention and treatment of colorectal cancer (7, 33, 34), as inflammation has key roles in tumor initiation, promotion, and metastasis (35). In fact, SDX-308, another NSAID agent, has been shown to impair the multiple myeloma cell proliferation and osteoclast activity by inhibiting the NF-κB activity signaling (31). On the other hand, De Luna-Bertos found that therapeutic doses of NSAIDs (dexamethasone, ketorolac, metamizole, and aspirin) modulated differentiation and antigenic profile activity of MG63 cells, whereas both dexamethasone and ketorolac inhibited osteoblast growth by arresting the cell-cycle and inducing apoptosis, which are influenced by the dose, duration of treatment, and cells used (36–39). These results indicate that the effects of these different NSAIDs are complicated in bone biology and/or osteosarcoma, and the differences in these data may be explained by the dose, treatment duration, and different NSAIDs used (40).

Strikingly, recent population studies support that aspirin reduces the risk of metastasis in colon cancer (7, 41). However, there is no evidence at the cellular levels or in the animal models to argue that aspirin may be benefit for treating cancer patients with potential metastasis. Consistently, during the preparation of our article, Ogawa and colleagues reported that aspirin reduced lung cancer metastasis to regional lymph nodes in vivo (42). Notably, the antitumor of aspirin is indefinite, as aspirin is protective in some cancer types, such as colorectal, gastric, liver, and prostate cancers (7, 10, 43), whereas aspirin is useless for some other cancer types (44–46). For randomized trials of aspirin, low dose and high dose is defined as 75 to 300 mg daily and 300 to 1,500 mg daily, respectively (47). In this report, the dose of aspirin we used was 100 mg/kg daily in mice, which is highly efficient to diminish the tumor growth and metastasis using the osteosarcoma orthotopic mouse model. The dose of 100 mg/kg daily in mice is equal to 8.13 mg/kg daily in humans, meaning that the aspirin level we tested is about 400 to 700 mg daily for humans weighing 50 to 80 kg. This dose of aspirin fits the definition of high-dose (300–1,500 mg daily) aspirin studied in previous randomized trials (47) and should provide adequate systemic exposure. At the same time, it should be noted that aspirin is used at 100 or 300 mg daily in the ongoing adjuvant aspirin trials for colorectal, breast, gastroesophageal, and prostate cancers (47).

In summary, we herein provide comprehensive evidence for an inhibitory effect of aspirin on osteosarcoma metastasis and demonstrate that aspirin enhances osteosarcoma chemotherapy. Because osteosarcoma is an extremely aggressive cancer type that lacks any targeted therapies, our findings strongly suggest that aspirin may be an excellent auxiliary drug for treating patients with osteosarcoma.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: D. Liao, T. Kang

Development of methodology: D. Liao, L. Zhong

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Liao, L. Zhong, T. Duan, X. Wang, G. Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Liao, L. Zhong, T. Duan, X. Wang, G. Wang

Writing, review, and/or revision of the manuscript: D. Liao, T. Kang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.-H. Zhang, K. Hu, X. Lv

Study supervision: T. Kang

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