Oncostatin M Induces Bone Loss and Sensitizes Rat Osteosarcoma to the Antitumor Effect of Midostaurin In vivo

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Abstract

Purpose: In cultures, the cytokine oncostatin M (OSM) reduces the growth and induces differentiation of osteoblasts and osteosarcoma cells into glial/osteocytic cells. Moreover, OSM sensitizes these cells to apoptosis driven by various death inducers such as the kinase inhibitor staurosporine. Here, we asked whether OSM would have similar effects in vivo.

Experimental Design: Adenoviral gene transfer of OSM (AdOSM) was done in naive and osteosarcoma-bearing rats, alone or in combination with Midostaurin (PKC412), a derivative of staurosporine currently used in cancer clinical trials. Bone variables were analyzed by microcomputed tomography scanner, by histology, and by the levels of various serum bone markers. Osteosarcoma progression was analyzed by the development of the primary bone tumor, evolution of pulmonary metastasis, histology (necrosis and fibrosis), and animal survival.

Results: In naive rats, AdOSM reduced serum osteoblastic and osteoclastic markers in correlation with a reduced trabecular bone volume. In an osteosarcoma rat model, the combination of AdOSM with PKC412 reduced the progression of the primary bone tumor, pulmonary metastatic dissemination, and increased overall survival, whereas these agents alone had no antitumor effect. Increased tumor necrosis and tissue repair (fibrosis) were observed with this combination.

Conclusion: These in vivo experiments confirm that systemic OSM overexpression alters osteoblast/osteosarcoma activity. Because OSM sensitizes rat osteosarcoma to apoptosis/necrosis, the use of kinase inhibitors such as Midostaurin in association with OSM could represent new adjuvant treatments for this aggressive malignancy.

Osteosarcomas are rare bone-forming tumors that affect primarily young adults. These cancer cells arise from osteoblasts, the cells responsible for bone apposition. Rather than a unique gene alteration, multiple deregulations of the proteins controlling the G1-S-phase cell cycle checkpoint (p53, Rb, p16INK4a, MDM2, etc.) are involved in the pathogenesis and chemoresistance of osteosarcomas (1, 2). Although the prognosis and chemotherapies of patients with osteosarcoma were improved significantly in the seventies, the survival rate after 5 years is only 60% to 70% and as low as 30% when pulmonary metastases are detected at diagnosis. These survival rates were not further ameliorated during the past decades (3, 4).

Thus, new therapies that inhibit the growth or metastasis of these tumors will have a significant effect on patient survival. Cytokines of the interleukin-6 (IL-6) family, such as oncostatin M (OSM), are recognized as pleiotropic factors influencing many pathophysiologic events in several organs, including bone (5–8). These cytokines have all been reported to stimulate osteoclastogenesis and, in some cases, to stimulate osteoblast differentiation in cell cultures. However, recent in vivo and genetic data have challenged these concepts (reviewed in refs. 5–8). For example, transgenic mice overexpressing IL-6 showed a decrease in osteoblast and osteoid as well as a decrease (9) or increase (10) in osteoclast and bone resorption.

Overexpression of OSM using adenoviruses in mouse joints induced inflammation, cartilage, and bone destruction (11), in association with increased osteoclast number (12), but also...
induced periosteal bone apposition (13). However, in vitro OSM appeared to have a dual effect on osteoblast differentiation. Thus, various reports indicated that OSM enhances osteoblast marker expression and bone nodule formation on osteoblast precursors or on the cell line MG63, a p53-negative osteosarcoma (14–16). In correlation, OSM blocks MG63 cells in the G1-G2 phase of the cell cycle by inducing the cyclin-dependent kinase inhibitor p21WAF1 (16). However, other reports on normal or transformed osteoblasts have shown an inhibitory effect of OSM on bone formation and markers expression in vitro (14, 15). It is possible that these dual effects depend on the differentiation stage of the osteoblast or osteosarcoma cells: on precursor cells, OSM would stimulate the first stages of differentiation, but on more mature cells it would stimulate the terminal differentiation into osteocytes characterized by reduced marker expression/bone formation/proliferation and enhanced apoptosis (15). Indeed, we observed that OSM alone does not induce cell death, but wild-type p53 osteoblastic or osteosarcoma cells treated with OSM are particularly sensitive to apoptosis induced by staurosporine (STS), UV, or tumor necrosis factor-α (17). OSM, through activation of STAT5 and p53, increases the Bax/Bcl2 ratio, which controls the mitochondrial apoptotic pathway, but concomitantly activates antiapoptotic signals through the kinases protein kinase Cδ and phosphatidylinositol 3-kinase/Akt (17).

Altogether, these results suggest that IL-6-type cytokines, especially OSM, could have opposite or dual effects on bone formation and resorption depending on the model, the pathophysiologic condition, and the presence of other inflammatory cytokines. Not surprisingly, these cytokines have also been described as pro-bone or anti-bone cancer agents. As a consequence, clinical trials were done either to use these cytokines as anticancer adjuvants (18) or to neutralize their unwanted effects on tumor growth and osteolysis (19). In fact, IL-6-type cytokines are known as potent growth inhibitors or stimulators depending on the model, the differentiation stage of the osteoblast or osteosarcoma cells to apoptosis, this cytokine could represent a new adjuvant treatment for this aggressive malignancy. Moreover, in vivo recombinant IL-6 and OSM mediates tumor regression of various sarcomas and carcinomas that form lung or liver metastases (20, 21), but there are no reports describing a similar effect on bone metastases or primary bone cancers. In contrast, several reports indicated that IL-6-type cytokines that are produced in the bone-tumor environment, mainly IL-6, interleukin-11, and leukemia inhibitory factor (LIF), participate in bone resorption and in tumor proliferation in bone (22–24). Therefore, they are believed to participate to the vicious cycle that takes place between bone tumor proliferation and associated inflammation and osteolysis (25, 26).

In this study, we sought to determine the role of OSM overexpression in vivo on osteosarcoma development and associated bone remodeling. In rats, OSM appeared to act as an inhibitor of both osteoblast and osteoclast activities, modulating bone turnover in normal or cancer situations. Moreover, in vivo OSM sensitizes osteosarcoma cells to the antitumor effect of Midostaurin (PKC412), a derivative of

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**Materials and Methods**

**Cell viability.** The rat osteosarcoma OSRGA cell line, derived from the osteosarcoma used in the present study, was described previously (15) and cultured in DMEM (BioWhittaker) supplemented with 5% fetal bovine serum. Cells were plated into 96-well plates at an initial density of 1,500 per well and cultured with recombinant mouse OSM, human IL-6, human soluble IL-6 receptor (IL-6Rs; R&D Systems), human LIF (a kind gift from Dr. A. Godard, INSERM U601), STS (Sigma), or Midostaurin (PKC412; N-benzoyl-STS; kindly provided by Dr. J. Roesel, Novartis Pharma) before adding XTT reagents (Roche Molecular Biomedicals). After incubation for 5 h at 37°C, the absorbance was read at 490 nm. All assays were done in triplicate.

**Caspase-3 activity.** Subconfluent OSRGA cell cultures in 24-well plates were treated as indicated with OSM, STS, PKC412, methotrexate (Calbiochem), doxoruicin (Sigma), mafosfamide (kindly provided by Dr. A. Martinez, Baxter Oncology), or cisplatin (Calbiochem) and then lysed in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L Na3VO4, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF, 10 µg/ml leupeptin, 10 µg/ml aprotinin]. Lysates were cleared of debris by centrifugation at 12,000 × g for 15 min. Caspase-3 activity was assessed on 10 µl total cell lysates using the kit CaspACE Assay System, Fluorometric (Promega) as indicated previously (17). Results were expressed in arbitrary units and corrected for protein content as determined by the BCA kit (Sigma).

**Cell motility and adhesion.** For cell motility, OSRGA cells were cultured in six-well plates until confluent and treated or not with OSM (100 ng/ml). A slit was then done on the cell monolayer and phase-contrast photographs were taken using a Leica microscope every 24 h. For cell adhesion, OSRGA cells were treated for 3 days with OSM (100 ng/ml), dissociated with PBS-EDTA (BioWhittaker), washed twice with DMEM containing 5% FCS, and plated into 96-well plates coated with collagen I, vitronectin, fibronectin, or Matrigel matrix (all from BD Biosciences) at an initial density of 2·10⁴ cells per well. After indicated times, cells were washed with PBS to eliminate nonadherent cells. Then, XTT reagents (Roche Molecular Biomedicals) were added to each well and incubated for 5 h at 37°C; absorbance was read at 490 nm using a 96-multimicrowell microplate reader. All assays were done in triplicate.

**In vivo treatment with the AdOSM.** Replication-deficient adenovirus encoding mouse OSM has been described previously (29) and was produced, together with adenosivirus encoding green fluorescent protein (AdGFP), in the vector facility of the INSERM U649 Laboratory. Seven-week-old male Sprague-Dawley rats (IFFA-CREDO) were injected i.v. in the portal vein or i.m. in the tibial anterior muscle with increasing doses of adenovirus as described (30). Animals were weighted twice a week until they showed signs of morbidity, at which point they were sacrificed by cervical dislocation. All experiments with adenoviruses were approved by the French Ministry of Research and were done in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators.

**Rat osteosarcoma model.** This model has been described in detail previously (31). Briefly, 5-week-old male Sprague-Dawley rats (IFIA-CREDO) were injected i.v. in the portal vein or i.m. in the tibial anterior muscle with increasing doses of adenovirus as described (30). Animals were weighted twice a week until they showed signs of morbidity, at which point they were sacrificed by cervical dislocation. All experiments with adenoviruses were approved by the French Ministry of Research and were done in accordance with the institutional guidelines of the French Ethical Committee and under the supervision of authorized investigators.

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**Adjuvant Treatment of Osteosarcoma with AdOSM**
diameters, respectively. Lung tumor dissemination was assessed at necropsy. The cumulative rate of overall survival (event-free survival) was calculated according to actuarial method and the endpoint considered was either death of animals or signs of morbidity, which included cachexia, respiratory distress, and/or animals bearing too bulky tumors (tumor volume >10,000 mm³).

**OSM reverse transcription-PCR.** At necropsy, the muscle and liver tissues were collected, used for total RNA extraction, and subjected to cDNA synthesis as described previously (15). For the PCR, the primers used were 18S sense 5'-TCAAGAACGAAAGTCGGAGGTTCG-3', 18S antisense 5'-TTATTGCTCAATCTCGGGTGGCTG-3', OSM sense 5'-ATATGCGGCCGCGCCTGGCTGCTCCAAC-3', and OSM antisense 5'-GACATGAGTCATTACCCGGCCGTGGTGTTGGACCC-3'. The thermal cycle profile was as follows: denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. For 18S and OSM, 23 and 40 cycles were done, respectively. PCR products were size fractionated on a 1% ethidium bromide/agarose gel and visualized under UV transillumination.

**Serum bone markers.** Blood was drawn intermittently from the retro-orbital vein to monitor various serum bone variables. Alkaline phosphatase activity was assayed using p-nitrophenyl phosphate as a substrate (Biome`rieux). Osteocalcin levels were estimated using the Rat-MID Osteocalcin ELISA (Nordic Bioscience Diagnostics). COOH-terminal telopeptides of type I collagen were quantified using the RatLaps ELISA (Nordic Bioscience Diagnostics). Osteoclast-derived tartrate-resistant acid phosphatase form 5b was measured using the RatTRAP Assay (SBA Sciences).

**Microscanner and radiologic analyses.** At necropsy, the right tibia was fixed in 10% buffered formaldehyde and dissected to discard soft tissues, comprising the major part of the tumor if present. Analysis of architectural variables was done using the high-resolution X-ray micro-computed tomography (micro-CT) system for small animal imaging SkyScan-1072 (SkyScan). Relative volume (BV/TV) of the tibia (cortical or trabecular bone) was quantified in the groups that received AdOSM compared with that of control rats. Other trabecular variables (number, thickness, and space) were similarly obtained using the SkyScan CtAn software. Radiographs on anesthetized animals (Nesdonal, 50 mg/kg) were considered significant.

**Histologic analysis.** Right tibia were fixed in 10% buffered formaldehyde and decalcified by electrolysis, and after embedding in paraffin, 5-μm-thick sections were mounted on glass slides. Transversal sections obtained from the tibia area were stained with H&E-safran. Sections obtained from the tumor tissues were stained with Masson trichrome. Analysis and quantification of necrotic and fibrotic areas was done using a Leica Q500 image analysis system.

**Statistics.** Statistical significance between the various animal groups was evaluated using the unpaired Mann-Whitney test. The differences of actuarial survival were determined by the K² test. Results with P < 0.05 were considered significant.

## Results

**OSM sensitizes rat OSRGA cells to apoptosis induced by Midostaurin in vitro.** We recently described that OSM inhibits proliferation and sensitizes rat OSRGA osteosarcoma cells to apoptosis driven by various death inducers such as STS, LIF, or tumor necrosis factor-α (15, 17). As shown in Fig. 1A, 3-day treatment with OSM (100 ng/mL) or IL-6 in combination with IL-6Rs significantly reduced the number of viable cells, whereas LIF or IL-6 alone had no effect. Because there was no induction of cell death with these cytokines (Fig. 1C; data not shown), it could be assumed that these reductions were largely due to an inhibition of cell proliferation by OSM or IL-6 + IL-6Rs as described previously (15, 17).

Whereas OSRGA cells were largely resistant to STS, pretreatment for 3 days with OSM sensitized them to cell death induced by low doses of STS (IC₅₀ = 0.1 μmol/L; Fig. 1B). The reduced number of viable cells was indeed related to cell death because it was associated with membrane blebbing and nuclear condensation (see below). Dose-response experiments indicated that 10 ng/mL OSM were necessary to observe a maximum effect on cell death (data not shown). In contrast, pretreatment with IL-6 did not sensitized to cell death, whereas LIF pretreatment induced only a slight increase of death (Fig. 1B). IL-6 plus IL-6Rs sensitized OSRGA cells to cell death induced by STS to the same level as observed with OSM. Altogether, these results indicated that, within the IL-6 family of cytokines, OSM was a major inducer of OSRGA cell death and that these cells
were not responsive to IL-6 presumably because they did not express the IL-6R at the cell surface, thus needing IL-6Rs to increase cell death.

Because STS is highly toxic in vivo, we then asked whether OSM would similarly synergize with Midostaurin, a well-tolerated STS-related drug (27, 28). As shown in Fig. 1B (right), OSRGA cells were resistant to PKC412, but pretreatment with OSM sensitized them to cell death induced by PKC412 (IC_{50} = 1 μmol/L). Decreased viability induced by OSM + PKC412 was associated with membrane blebbing and nuclear condensation (data not shown) as described previously with OSM + STS, suggesting an induced apoptosis. Indeed, both OSM + STS and OSM + PKC412 induced caspase-3 activity in OSRGA cells, but again we noticed that a 10-fold excess of PKC412 was needed to reach the level obtained with STS (Fig. 1C).

Polychemotherapy of osteosarcoma relies on methotrexate, ifosfamide, doxorubicin, and cisplatin regimens, some of these drugs being well-known to induce apoptosis in cancer cells through the mitochondrial apoptotic pathway (4, 32, 33). OSM also sensitized OSRGA cells to cell death induced by doxorubicin or cisplatin as revealed by the enhanced caspase-3 activity (Supplementary Fig. S1A and B). In contrast, we could not observe cell death when combining OSM with methotrexate or mafosfamide, the active compound of ifosfamide (ref. 34; Supplementary Fig. S1C and D).

**OSM enhances motility and reduces adhesion of OSRGA cells in vitro.** The role of OSM on cancer metastasis is controversial. This cytokine could enhance the metastatic capacity of breast cancer cells (35) but reduces migration and invasion of lung cancer cells (21). As shown in Supplementary Fig. S2, OSM enhances motility of OSRGA osteosarcoma cells. In addition, OSM reduces adhesion to several matrix proteins such as vitronectin (Supplementary Fig. S3A), fibronectin, or collagen I (data not shown). A similar inhibitory effect of OSM was observed on reconstructed basement membrane gel (Matrigel-coated plates; Supplementary Fig. S3B). These results suggest that OSM could have a significant effect to enhance the metastatic potential of OSRGA cells.

**High doses of OSM are toxic in rats.** To assess the effect of OSM in vivo, injections of an adenovirus encoding OSM (AdOSM) were administered i.v. or i.m. (tibial anterior muscle) to male Sprague-Dawley rats. The AdOSM appeared to be highly and dose-dependently toxic when delivered i.v., with $5 \times 10^9$ plaque-forming units (pfu) AdOSM inducing 90% of mortality within 7 days (Fig. 2A). Animals receiving the AdOSM i.v. rapidly lost weight (for the highest doses) or had a severely reduced gain of weight (for the lowest doses; Fig. 2B). In fact, during the first week postinjection of the high doses of AdOSM, rats harbored flu-like symptoms, with conjunctivitis and generalized inflammation (data not shown). These symptoms were never observed with the control virus encoding GFP (Fig. 2A and B). Similar amount of AdOSM injected i.m. were well tolerated, with no mortality observed (Fig. 2A), only transient and reversible weight loss (Fig. 2B), and only local inflammation at the site of virus injection (data not shown).

In the absence of specific ELISA for mouse OSM, the mRNA level of this cytokine was then analyzed by reverse transcription-PCR on various tissue samples (Fig. 2C). OSM transcripts were found expressed only in the liver after i.v. injections and only in the tibial anterior muscle after injection in this tissue. Moreover, mRNA expression levels were higher in the liver than in muscles, suggesting that the reduced toxicity observed after the i.m. injections is related, likely in part, to reduced circulating levels of OSM. Whatever the reasons, further experiments especially in the rat osteosarcoma model (see below) were done with i.m. injections of AdOSM.

**AdOSM induces trabecular bone loss.** Seven days after AdOSM injections, the serum levels of various bone markers were analyzed (Fig. 3A and B). Serum alkaline phosphatase levels were dose-dependently reduced by OSM whether the AdOSM was injected i.v. or i.m. (Fig. 3A). Similarly, serum osteocalcin levels were reduced by OSM (Fig. 3A), suggesting a reduced osteoblast activity. Both serum tartrate-resistant acid phosphatase form 5b and COOH-terminal telopeptide of type 1 collagen levels, markers of mature osteoclasts and bone resorption, respectively, were also reduced in rats injected with the AdOSM (Fig. 3B).

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![Fig. 2. Toxicity of the AdOSM in naive rats. Rats were injected in the portal vein (i.v.) or in the tibial anterior muscle (i.m.) with the indicated dose (in pfu) of AdOSM or AdGFP. A, mortality at day 7 postinjection (6-10 animals per group corresponding to three different experiments). B, evolution of weight (4 animals per group). CT, control group of animals injected with $5 \times 10^9$ pfu AdGFP i.v. C, 7 d postinjection, animals were sacrificed and liver and tibial anterior muscle tissues were analyzed by reverse transcription-PCR with primers specific for the indicated cDNA.](image-url)
At the tibia level, micro-CT scanner analysis revealed that OSM does not modify the cortical bone volume (data not shown) but significantly reduces the trabecular bone volume (Fig. 3C). Trabecular number was significantly reduced by OSM, whereas the trabecular space increased. Trabecular thickness was not significantly altered (Fig. 3C). These results were observed in the tibia near the injection site or in the contralateral tibia or after i.v. injection (Fig. 3D; data not shown), indicating a systemic effect of OSM on bone remodeling.

**Combination of AdOSM with Midostaurin limits osteosarcoma progression and enhances survival.** A rat osteosarcoma model was then used corresponding to the OSRGA cell line. Tumor fragments were transplanted in close contact to the tibiae surface and AdOSM was injected i.m. in the contralateral tibial anterior muscle. OSM was unable to modify the development of the primary bone tumor or the metastatic pulmonary dissemination (Fig. 4A–C). However, because in vitro pretreatment with OSM sensitizes OSRGA cells to apoptosis induced by Midostaurin (PKC412; Fig. 1), the potent antitumor effect of this combination was analyzed in our rat osteosarcoma model. Preliminary experiments indicated that oral administrations of PKC412 up to 30 mg/kg/d were not toxic and did not exert any effect on tumor development (Fig. 4A–C; data not shown). However, when associated with the AdOSM, PKC412 reduced tumor progression. In a first set of experiments, primary bone tumor volumes were reduced 2.5-fold by OSM + PKC412 32 days after tumor implantation (18 days after AdOSM injection; Fig. 4A). These results were confirmed in subsequent experiments with, on average, a relative tumor volume reduced by 2-fold ($P = 0.01$; Fig. 4B). At the time of sacrifice, we also noticed a 2-fold reduction in the incidence of lung metastasis for the animals treated with AdOSM and PKC412 (27% versus 50% for the control group; Fig. 4C). As a result of the decreased tumor

**Fig. 3.** AdOSM reduces serum bone markers expression and induces trabecular bone loss. A, rats were injected i.v. or i.m. with AdOSM or AdGFP at the indicated dose ($\times 10^6$ pfu). Seven days later, sera were collected and analyzed for alkaline phosphatase activity (ALP; filled columns) or osteocalcin level (OC; hatched columns; $n = 8$, except for the group AdOSM at $2.5 \times 10^6$ pfu in which $n = 4$ because of the mortality). B, rats were injected i.m. with $1 \times 10^6$ pfu adenoviruses. Seven days later, they were analyzed for serum tartrate-resistant acid phosphatase form 5B (TRAP5B; filled columns) or COOH-terminal telopeptide of type I collagen (CTX; hatched columns; $n = 8$ in each group). C, rats were injected i.m. with $1 \times 10^6$ pfu AdOSM or AdGFP. D, rats received the adenoviruses either i.v. or i.m. as indicated. Seven days later, bone variables of the tibiae were analyzed by micro-CT scan. C, top, sagittal sections; bottom, relative trabecular bone variables calculated from three-dimensional image registration data. BV/TV, relative trabecular bone volume; TB.TH, trabecular thickness; TB.N, trabecular number; TB.SP, trabecular space. D, relative trabecular bone volume ($n = 3$ in each group). Columns, mean; bars, SD. a, $P < 0.05$, compared with the control AdGFP group.
burden, the overall survival was significantly increased after treatment with OSM + PKC412 (Fig. 4D). In the groups treated with AdOSM or PKC412 alone, primary tumor progression (Fig. 4A and B), metastatic pulmonary incidence (Fig. 4C), or overall survival (Fig. 4D) was not modified.

**AdOSM alters the ectopic bone neoformation in the rat osteosarcoma model.** To better describe the modifications induced by OSM and potently related to the sensitization to PKC412, we next analyzed the effect of OSM overexpression on cancer-induced bone remodeling and on cells implicated in this process. In this animal model, development of the tumor is known to induce osteolysis as well as an important ectopic bone formation at the tibia surface (Fig. 5A and B; ref. 31). This extensive bone remodeling induced by osteosarcoma cells is thought to participate to tumor development and is related to activation of both periosteal osteoblasts and osteoclasts, which appeared numerous in this ectopic bone area (Fig. 5B). Radiography and micro-CT scanner analysis revealed that this periosteal bone apposition has a well-organized and localized, sunshine appearance (Fig. 5A and C, arrows), characteristic of osteosarcoma-associated bone lesions. The AdOSM alone altered this ectopic bone formation, which appeared more anarchic, dispersed all over the tibia, and did not adopt a radiant structure anymore (Fig. 5C, arrows), indicating alterations in bone remodeling by OSM. However, histology in these ectopic bone areas did not revealed important modifications induced by AdOSM on the osteoblastic/osteocytic, osteoclastic, or tumoral contingents (data not shown).

**AdOSM + PKC412 induces necrosis and fibrosis in the tumor tissues.** Histologic analyses in the tumor tissues showed that the decreased primary tumor volume in the group of animals treated with OSM + PKC412 was associated with an increased tumor necrosis, especially at the cutaneous surface of the tumors (Fig. 6D, asterisks) compared with other groups (Fig. 6A–C). Quantification of the necrosis areas indicated a 3-fold increase in the OSM + PKC412 group (from 13%, 20%, and 18% of necrosis in the AdGFP, AdGFP + PKC412, and AdOSM groups, respectively, to 60% in the AdOSM + PKC412 group). Moreover, the collagen Masson trichrome staining revealed an important fibrotic component in the tumors treated with OSM + PKC412, with striking figures of fibrosis surrounding tumor nodules (arrows in Fig. 6D, inset). Fibrosis was also observed inside the tumor nodules as well as in necrotic areas, suggesting an induced tissue repair in comparison with the control, OSM, or PKC412 groups (Fig. 6A–D). With the association OSM + PKC412, we also observed an induced lymphocytic and polynuclear reaction in these superficial necrotic and fibrotic regions (data not shown).

**Discussion**

In vitro, OSM is known to induce differentiation, to inhibit proliferation, and/or to enhance apoptosis of various cancer cell lines (derived from melanomas or osteosarcomas for example), suggesting a therapeutic application for this cytokine. Within the IL-6 family of cytokines, OSM has been shown to possess the strongest anticancer effects, for example, on
proliferation (16) or apoptosis (Fig. 1A), followed by IL-6 but only in combination with its soluble receptor. This process, termed IL-6 trans-signaling, allows cells that do not express the IL-6R to respond to this cytokine (6–8). In bone, IL-6Rs is required for IL-6 effects on osteoblasts (6–8) and both IL-6 and IL-6Rs are required to lower the rate of melanoma growth in mice (36). Similarly, recombinant OSM reduces melanoma progression in mice, especially lung metastasis (14), but there are no data concerning its potent role on cancer progression in the bone environment. IL-6-type cytokines such as OSM have been shown to induce bone resorption in various experimental settings (12, 13, 37) and to participate in osteolysis associated with cancer development in bones (22–24). Bone degradation then allows the release of growth factors, further enhancing tumor proliferation in bones (25, 26). Here, we show that OSM alone is not sufficient to limit osteosarcoma proliferation in the bone environment, but that in combination with Midostaurin it significantly reduced primary bone tumor development and metastasis to lungs and enhances overall survival. Thus, we show for the first time that OSM could be considered as an adjuvant treatment for bone tumors such as osteosarcoma. Whether IL-6, especially in combination with its soluble receptor, would have similar effects on bone cancer is currently under investigation.

The osteosarcoma model presented here is in rodents; to the best of our knowledge, murine OSM used in this study does not bind to the LIF receptor (gp130 + LIFRα) in contrast to human OSM (38). Therefore, the effects of murine OSM are expected to be mediated exclusively through its specific receptor (gp130 + OSMRβ); indeed, we observed that OSRGA cells express gp130 and OSMRβ mRNA as well as LIFRα mRNA. Because LIF mildly but significantly sensitized OSRGA cells to apoptosis (Fig. 1B), we can assume that the receptor for LIF (gp130 + LIFRα) also sensitizes OSRGA cells to apoptosis and thus could also synergize with Midostaurin in vivo but to a lower extent than the receptor for OSM.

That OSM alone has no anticancer effect in the osteosarcoma model presented here is surprising because this cytokine readily inhibits proliferation and induces expression of the cyclin-dependent kinase inhibitor p21WAF1 in vitro (15). However, we noticed previously that the growth-inhibitory effect of OSM on these cells is only observed in serum-free or low-serum conditions (15), suggesting that serum growth factors could prevent the inhibitory effect of OSM in vitro and also in vivo. OSM similarly inhibits the growth of osteosarcoma cell lines, such as ROS17/2.8, MG63, or SaOS2 cells, by blocking the cells in the G1 phase of the cell cycle, but these effects are not prevented by serum (15–17, 39). Therefore, other animal models are needed to confirm the hypothesis that OSM alone could also prevent osteosarcoma progression in vivo by inhibition of cancer cell proliferation.

Based on previous studies, it was also possible that OSM alone or in combination with PKC412 alters metastasis of cancer cells. Indeed, we observed that OSM enhances motility...
of OSRGA cells and reduces adhesion to several matrix components such as vitronectin, fibronectin, or collagen I, suggesting an enhanced metastatic potential as described previously for breast carcinoma cells (35). Similarly, the reduced cell binding to basement membrane (Matrigel) could limit adherence or readherence in a particular environment, thus promoting migration to a secondary growth site. However, OSM does not modify expression of matrix metalloproteinases potently implicated in metastasis but induces expression of TIMP1, an inhibitor of several matrix metalloproteinases.\(^5\) Similarly, systemic OSM enhances TIMP1 expression in the lungs of mice (29) and reduces pulmonary melanoma metastasis (21), suggesting that OSM could indeed limit osteosarcoma metastasis to the lung. In the rat osteosarcoma model presented here, systemic OSM alone does not modify the metastatic potential, but in combination with Midostaurin it significantly reduces lung metastasis.

Previous studies indicated that OSM exerts a dual effect on osteoblast differentiation, stimulating the first stages of osteoblastogenesis or inducing terminal differentiation into osteocytes depending on the differentiation stage of the cells used (14, 15). In addition, OSM has been shown to induce RANKL expression by osteoblastic or stromal cells, thus supporting osteoblastogenesis and bone resorption (12, 13, 39). However, other studies revealed a potent inhibitory effect of IL-6-type cytokines on osteoclast formation and bone resorption (9, 40). Recently, Sims et al. observed that OSM receptor-deficient mice have both a reduced bone formation by osteoblasts and a significantly reduced bone resorption by osteoclasts (41), indicating that OSM, or other cytokines using the OSM receptor such as interleukin-31 (42), have an important role in maintaining normal levels of bone remodeling. Here, we observe that systemic OSM overexpression reduces serum osteoblastic and osteoclastic markers as well as the trabecular bone volume in naïve rats. We cannot exclude the possibility that OSM first stimulated osteoblastogenesis and bone resorption (within few days) and later inhibited these variables (within 1 week), but our results clearly indicate that OSM inhibits osteoblast activity in vitro (15) and in vivo (this study). Bone loss induced by OSM could thus be related in part to reduced bone formation by osteoblasts. Moreover, considering that osteoblasts are necessary to support osteoclast formation, it is possible that the reduced osteoblastic activity induced by OSM indirectly also reduced osteoclast activity. In contrast to the net inhibitory effect of OSM overexpression, it seems that physiologic levels of endogenous OSM regulate maintenance of bone remodeling, because OSM receptor deficient mice have decreased bone formation and resorption (41).

Similarly, OSM alters the ectopic bone formed at the tibia surface and at the contact of osteosarcomas, bone neoformation being more anarchic and dispersed all around the tibia. These alterations did not appear to depend on induced bone resorption as (a) there was no modification in the number of osteoclasts in the ectopic bone areas and (b) alterations were still observed in the presence of zoledronate, a powerful inhibitor of osteoclast-mediated osteolysis.\(^5\) Moreover, because OSM overexpression alone did not modify osteosarcoma progression in our model, we can conclude that the potent osteolytic effect of this cytokine, which was not observed in this study, was in any case not sufficient to stimulate the vicious cycle between bone resorption and tumor proliferation. Whether the alteration in ectopic bone induced by OSM is linked to an induced terminal differentiation of osteoblasts/osteosarcoma cells into osteocytic cells deserves additional experiments, but they are clearly associated with sensitization of the tumor cells to the anticancer agent Midostaurin (see below). Altogether, these results indicate that overexpression of OSM alone in vivo alters bone formation in physiologic or cancer-associated situations but with no significant effect on cancer progression.

The mechanism by which OSM plus Midostaurin (PKC412) reduces osteosarcoma progression in rats seems to rely on a direct induction of apoptosis in cancer cells. Indeed, we observed an induced necrosis with the combination of OSM with PKC412 in vivo as well as a higher proportion of fibrotic tissue and immune cell infiltration. There was no modification in terminal deoxynucleotidyl transferase–mediated dUTP nick

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**Fig. 6.** Combination AdOSM + PKC412 induces necrosis and fibrosis in osteosarcoma tumor tissue. Rats were implanted with tumor fragments and treated with adenoviruses and PKC412 as described in Fig. 4. Masson trichrome staining was done on the tumor tissue (see Fig. 4A) near the cutaneous surface at the time of sacrifice (34 d postimplantation). A, AdGFP + placebo animal group (n = 2); B, AdGFP + PKC412 animal group (n = 2); C, AdOSM + placebo animal group (n = 2). D, AdOSM + PKC412 animal group (n = 3). The combination AdOSM + PKC412 induced both necrosis (asterisk, clear areas) and fibrosis (arrows, green areas). Representative section is shown for each group. Original magnification, ×20 (left) or ×120 (insets, right).
end labeling staining (apoptosis) presumably because our histologic analyses were done at the endpoint of the experiments (34 days postimplantation) when the expression of OSM is largely reduced (as described previously; ref. 29). However, the necrosis (or late apoptosis) observed at that time point in vivo together with the apoptosis observed in vitro strongly suggests that the combination of OSM with PKC412 induces apoptosis and then necrosis in these osteosarcoma cells in vivo. Previous studies have also reported an induced fibrosis (through extracellular matrix production and fibroblast proliferation) and recruitment of immune cells (through chemokine production) by OSM in the lungs (27, 43, 44) and/or joints of mice (11–13) that could explain part of the proinflammatory and repair reactions observed in our cancer model.

As described previously in vitro (17), it is therefore possible that OSM in vivo, through the activation of p53 and STAT3, increases the expression of Bax and reduces the expression of Bcl2, thus sensitizing the mitochondria to suboptimal doses of death inducers in osteosarcoma cells. When PKC412 is given to rats, Bax is translocated from the cytosol to the mitochondria in cancer cells, activating the mitochondrial apoptotic pathway. However, OSM also induces activation of various survival kinases such as protein kinase Cβ, Akt, and extracellular signal-regulated kinase 1/2, and we described previously that STS inhibits their activation by OSM (17). Therefore, an additional mode of apoptosis induction by STS or its derivative Midostaurin (PKC412) could be mediated through inhibition of survival kinases. In line with these data, PKC412 alone is highly active on tumors such as leukemia with FLT3 receptor mutations by directly inhibiting this tyrosine kinase (45). In contrast, several fibrosarcoma and adenosarcoma and the OSRGA osteosarcoma appear resistant to PKC412 both in vitro and in vivo (17, 46). In these cells, however, combination of PKC412 and other anticancer treatments such as irradiation or OSM proves to be particularly active to induce, in a p53-dependent manner, the mitochondrial apoptotic pathway and destruction of cancer cells in vivo (17, 46).

Collectively, our results suggest that an adjuvant treatment with OSM could sensitize osteosarcoma cells to apoptosis/necrosis induced by Midostaurin. Therefore, there may be other new, still unknown cancer indications for this kinase inhibitor and further investigations are needed to adequately describe the list of cancer cells sensitive to this new bi-therapy. Because OSM can alter several steps in the complex process of metastasis, a careful examination of its potent unwanted effects in adequate cancer models is necessary. In addition, polychemotherapy of osteosarcoma actually comprise methotrexate, ifosfamide, doxorubicin, and cisplatin regimens (4), and OSM sensitizes OSRGA osteosarcoma cells to cell death induced by two of these drugs, doxorubicin and cisplatin. Similar to STS or Midostaurin, doxorubicin and cisplatin are known to induce cell death in osteosarcoma through p53 and the mitochondrial pathway (32, 33). Therefore, an adjuvant treatment with OSM could synergize with conventional chemotherapy or will be able to sensitize resistant tumors, such as advanced metastatic osteosarcomas, offering new lines of therapeutic interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Oncostatin M Induces Bone Loss and Sensitizes Rat Osteosarcoma to the Antitumor Effect of Midostaurin \textit{In vivo}

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