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Mesenchymal Stem Cells Induce Resistance to Chemotherapy through the Release of Platinum-Induced Fatty Acids

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SUMMARY

The development of resistance to chemotherapy is a major obstacle for lasting effective treatment of cancer. Here, we demonstrate that endogenous mesenchymal stem cells (MSCs) become activated during treatment with platinum analogs and secrete factors that protect tumor cells against a range of chemotherapeutics. Through a metabolomics approach, we identified two distinct platinum-induced polyunsaturated fatty acids (PIFAs), 12-oxo-5,8,10-heptadecatrienoic acid (KHT) and hexadeca-4,7,10,13-tetraenoic acid (16:4(n-3)), that in minute quantities induce resistance to a broad spectrum of chemotherapeutic agents. Interestingly, blocking central enzymes involved in the production of these PIFAs (cyclooxygenase-1 and thromboxane synthase) prevents MSC-induced resistance. Our findings show that MSCs are potent mediators of resistance to chemotherapy and reveal targets to enhance chemotherapy efficacy in patients.

INTRODUCTION

Chemotherapy remains the primary treatment for most disseminated cancers. However, the response to chemotherapy is often transient, and development of resistance is one of the most significant obstacles to effective cancer therapy. Although various tumor cell-intrinsic mechanisms of drug resistance have been identified, it is becoming increasingly clear that the tumor microenvironment plays a key role in the development of drug resistance (reviewed in Meads et al., 2009). Whereas acquired tumor cell-intrinsic resistance develops over time, environment-mediated drug resistance is rapidly induced by signaling events from the tumor microenvironment and is likely reversible because removal of the microenvironment restores the drug sensitivity (Meads et al., 2009). The important role of the microenvironment in therapy response is further

Significance

Chemotherapy remains the primary treatment for most disseminated cancers. However, response to chemotherapy is often transient, and the development of resistance is one of the most significant obstacles to effective cancer therapy. We now show that mesenchymal stem cells (MSCs) activated by platinum-based chemotherapy secrete two unique fatty acids that, in minute quantities, confer resistance to multiple types of chemotherapy. This highlights an undesired role for stem cells in cancer treatment and reveals a potent effect of two relatively unknown fatty acids. Finally, our results reveal a therapeutic approach to enhance the clinical benefit of chemotherapy by blocking the release of these fatty acids from MSCs via inhibition of cyclooxygenase-1 or thromboxane synthase.

demonstrated by the fact that specific properties of stromal cells in the tumor microenvironment are often an indicator of poor prognosis. For instance in breast cancer patients, the stromal gene expression within the tumor predicts resistance to preoperative chemotherapy (Farmer et al., 2009). The surrounding stroma is believed to modulate the response to chemotherapy by either direct cell-cell interactions with tumor cells, or by the local release of soluble factors such as interleukin-6, promoting survival and tumor growth (Weaver et al., 2002; Shekhar et al., 2007; Hazlehurst et al., 2003; Sethi et al., 1999; Müerköster et al., 2004).

Tumors actively modulate their microenvironment by recruiting inflammatory cells and bone marrow-derived cells (BMDCs) (Kaplan et al., 2005; Jain and Duda, 2003; Lyden et al., 2001). It was recently shown that BMDCs can act in an immediate "seek and repair" manner in response to therapy, presumably in order to support tissue regeneration (Shaked et al., 2008). A subgroup of these cells, mesenchymal stem cells (MSCs), has gained much interest as mediators of cancer progression. MSCs are multipotent cells capable of differentiating into numerous cell types, including adipocytes, osteoblasts, chondrocytes, fibroblasts, and perivascular and vascular structures (Pittenger et al., 1999; Beckermann et al., 2008; Schmidt et al., 2006; Ringe et al., 2007). Although MSCs are found predominantly in the bone marrow (BM), resident MSCs have been described in various organs, and a small population is retained in circulation (Young et al., 2001). The relevance of MSCs becomes apparent in cases of tissue repair, wound healing, and inflammation. In these conditions paracrine signaling leads to mobilization of MSCs from BM and subsequent recruitment to the damaged site (Tögel et al., 2005; Morigi et al., 2008; Chen et al., 2008). Importantly, MSCs are recruited in large numbers to the stroma of developing tumors. Growing tumors constantly produce paracrine and endocrine signals mobilizing MSCs from the BM (Kidd et al., 2009; Loebinger et al., 2009; Schmidt et al., 2006; Ringe et al., 2007). In the tumor, MSCs are found to stimulate tumor growth, enhance angiogenesis, and promote metastasis formation through the release of a large spectrum of growth factors and cytokines (Beckermann et al., 2008; Karnoub et al., 2007; Bergfeld and DeClerck, 2010; Rhodes et al., 2010). In this study we addressed if MSCs are also involved in the development of resistance to chemotherapy. We investigated whether MSCs, recruited to the circulation by solid tumors, may play a role in the development of resistance to multiple types of chemotherapy.

RESULTS

MSCs Induce Resistance to Chemotherapy

To test the hypothesis that MSCs confer resistance to chemotherapy, we established different murine tumor models in which we resembled the mobilization and recruitment of MSCs from the BM to tumor. MSCs were harvested from the BM of syngenic mice and injected intravenously (i.v.) in mice bearing a subcutaneous (s.c.) tumor. MSCs used were either freshly isolated from the BM or cultured for five to seven passages to obtain a pure MSC population before injection (Li et al., 2009; Pevsner-Fischer et al., 2007). The cultured MSCs were analyzed by FACS analysis, and the number of cfu-F was determined. Multilineage differentiation potential confirmed the true MSC phenotype (see Figures S1A and S1B available online). The recruitment of MSCs to the tumor after i.v. injections has previously been demonstrated in a variety of animal models (Wang and Dubois, 2010). Consistently, 4 days after i.v. administration of 100,000 MSCs derived from GFP-positive mice, we found GFP-positive cells specifically in the s.c. tumors (0.05%–0.1% of all cells). There was no detectable accumulation of MSCs in other organs such as lungs, kidney, spleen, and liver (Figure S1C). In line with published data (Karnoub et al., 2007), administration of MSCs did not alter the growth kinetics of the tumor (Figure S1D). However, when the mice were treated with a commonly used chemotherapeutic agent, cisplatin (6 mg/kg), we found that MSCs administered i.v. just prior to chemotherapy negated the antitumor effect of cisplatin in a dose-dependent manner (Figure 1A). Intravenous administration of as few as 50,000 MSCs abolished the antitumor effects of cisplatin and resulted in a tumor volume similar to the untreated controls (Figure 1A). These findings were reproduced in two independent, s.c. mouse tumor models (C26 colon carcinoma cells in BALB/c, and Lewis lung carcinoma [LLC] cells in C57/Bl6) (Figure 1B), and were obtained by using both freshly isolated MSCs and cultured pure MSCs. The size of the tumor did not affect the ability to induce resistance; in larger tumors a similar effect was seen (Figure 1C).

MSCs Induce Resistance in a Systemic Manner

Given our observation that MSCs induce resistance to cisplatin and the notion that only a small number of MSCs engrafted in the tumors, we hypothesized that the induction of chemoresistance is due to a paracrine or systemic effect instead of an effect requiring direct cell-cell interaction. To investigate this, we s.c. injected MSCs at a site distant from the tumor. Subcutaneous MSCs were even more potent in inducing resistance to cisplatin compared to the i.v. administered MSCs. As few as 1000 MSCs injected s.c. in the other flank of the mouse than the tumor could induce partial resistance to cisplatin chemotherapy (Figure 1D). To provide further support of a systemic effect, we established in vitro cultures of mouse MSCs and treated mice with daily s.c. injections of conditioned medium (CM) harvested from cultured MSCs. When we preincubated cultured MSCs with 1 μM cisplatin for as short as 30 min and incubated for only 1 hr with serum-free medium (SFM) and subsequently administered this CM+ to tumor-bearing mice at the start of systemic treatment with cisplatin, we observed complete resistance to cisplatin for both the C26 tumors and LLC tumors, whereas CM from untreated MSCs (CM−) had no effect on the in vivo response to cisplatin (Figures 1E and 1F). Together, these data show that MSCs are rapidly activated by cisplatin to release systemic factors that subsequently induce resistance to chemotherapy.

Platinum-Activated MSCs Confer Resistance to Various Chemotherapeutics

To determine whether other chemotherapeutic agents could elicit the same MSC-induced resistance response, we tested different types of chemotherapy in our tumor models. Interestingly, in vivo resistance was only induced by MSCs in combination with MTD administration of the platinum analogs cisplatin, oxaliplatin (10 mg/kg), or carboplatin (100 mg/kg), but not with

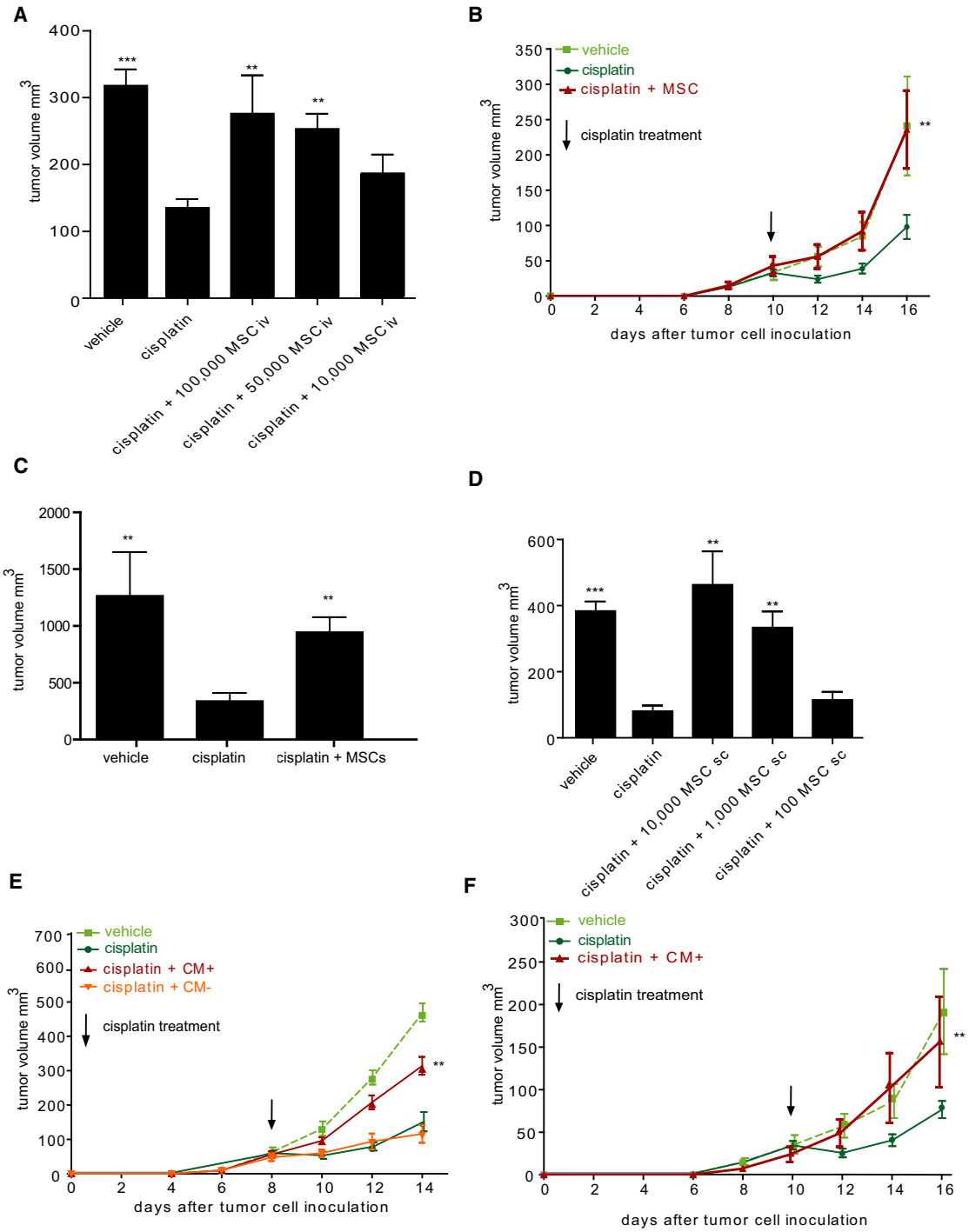

Figure 1. MSCs Induce Resistance to Cisplatin in a Paracrine Fashion

(A) Tumor volume of C26 cells inoculated s.c. in BALB/c mice 4 days after treatment with either vehicle control, cisplatin (6 mg/kg) or cisplatin plus coadministration of 100,000, 50,000, or 10,000 MSCs i.v. Treatment was initiated when tumors reached a volume of 50–100 mm^3.

(B) Tumor growth of LLC cells inoculated s.c. in C57Bl/6 mice, either untreated or treated with cisplatin alone or with cisplatin plus 100,000 MSCs i.v.

(C) Tumor volume of C26 cells 4 days after treatment with either vehicle control, cisplatin (6 mg/kg) or cisplatin plus coadministration of 50,000 MSCs i.v. Treatment was initiated when tumors reached a volume of 400–500 mm^3.

(D) Tumor volume of C26 cells 4 days after treatment with cisplatin with or without coadministration of MSCs s.c. in the other flank of the mouse (10,000, 1,000, or 100 MSCs).

(E) Tumor growth of C26 cells inoculated s.c. either untreated, treated with cisplatin alone or with cisplatin plus s.c. injections of 100 μl of the CM+ or CM−.

(F) Tumor growth of LLC cells either untreated, treated with cisplatin alone or with cisplatin plus s.c. injections of the CM+.

5-FU (100 mg/kg) or irinotecan (100 mg/kg) (Figure 2A). We next tested CM obtained from MSCs incubated with 1 μM of different types of chemotherapy. We found that only CM of MSCs preincubated with platinum analogs for 30 min followed by a serum free medium (SFM) incubation of 2 hr was able to induce resistance. The CM of MSCs preincubated with 5-FU, irinotecan, paclitaxel, or doxorubicin could not induce resistance in vivo (Figure 2B). Alternatively, we investigated whether platinum-activated MSCs could provide protection against other chemotherapeutic agents. Therefore, we treated tumor-bearing mice with either 5-FU or irinotecan in combination with daily s.c. injections of CM+ from cisplatin-preincubated cultured MSCs. Interestingly, we found that CM+ effectively induced resistance to both 5-FU and irinotecan (Figure 2C). To show that also the endogenous MSCs, present in the circulation of tumor-bearing mice, can be activated by platinum to secrete the resistance-inducing factors, we treated tumor-bearing mice with cisplatin, and after 30 min we harvested the MSCs from the circulation of these mice. These MSCs were subsequently injected s.c. into a recipient tumor-bearing mouse concomitantly with irinotecan treatment, which does not activate MSCs to produce the systemic factors in vivo. Strikingly, we found that indeed these MSCs induced resistance to irinotecan (Figure 2D), showing that endogenous, circulating MSCs are activated by platinum-based chemotherapy to secrete the resistance-inducing factors. Therefore, we concluded that MSCs need to be activated by a platinum-based chemotherapeutic agent in order to confer resistance and that the secreted factor(s) induces resistance against various types of chemotherapy. The fact that the secreted factor(s) induces resistance to very different types of chemotherapy suggests a general mechanism of protection of the tumor at the level of apoptosis.

To investigate whether the release of chemoprotective factors in response to platinum-based chemotherapy is a MSC-specific phenomenon, we tested different cell types for their ability to induce resistance in response to cisplatin. Hematopoietic stem cells (HSCs) and mature hematopoietic cells could not mimic the effects obtained with MSCs (data not shown). Next, we tested whether more differentiated mesenchymal cells could produce the protective factor(s) in response to cisplatin. We tested CM+ from mouse embryonic fibroblasts (MEFs), fibroblasts (3T3), preadipocytes (3T3-L1), differentiated adipocytes, preosteoblasts (MC3T3), and differentiated osteoblasts, and found that in addition to MSCs, only CM+ from MEFs could induce resistance in our mouse models. CM+ harvested from more differentiated cell lines did not induce resistance in vivo (Figure 2E). These findings indicate that only mesenchymal cells with a multilineage differentiation potential such as MSCs or MEF cells retain the capacity to secrete chemoprotective factors in response to platinum-based chemotherapy.

Systemic Induction of Resistance via Indirect and Reversible Prevention of Apoptosis

We showed that a single dose of the CM+ was sufficient to induce resistance. However, the CM+ had to be administered within 3 hr after cisplatin treatment to exert the protective effect (Figure 3A), indicating that the protective factors in the CM+ intervene with the early effects of chemotherapy. Interestingly, the tumor-protective effect exerted by CM+ was reversible because omitting the coadministration of the CM+ during a subsequent cycle of cisplatin restored the sensitivity to chemotherapy (Figure 3B). Therefore, we concluded that the secreted factor(s) did not induce persistent changes in the tumor but induced an acute and reversible protection of the tumor cells. Correspondingly, we did not find any changes in the amount of tumor stroma, microvascular density, or macrophages infiltration, evaluated by smooth muscle actin, desmin, vWf, F4/80 stainings, and rhodamine dextran injections (Figures S2A and S2B). However, both injection of MSCs and CM+ significantly reduced the number of apoptotic caspase-3-positive tumor cells by 50% to less than 2.5% ($p < 0.01$, Student's t test) in comparison to 5% in the tumors treated with cisplatin alone (Figure 3C). No difference in proliferation was observed as measured by Ki-67 (data not shown) or BrdU staining. (Figure 3D). The reduction in apoptosis is not due to a reduced exposure to chemotherapy. Similar chemotherapeutic exposure of the tumors was confirmed by pharmacokinetics (PK) of both cisplatin and irinotecan in plasma and tumor tissue. No differences were observed in the PK of cisplatin or in irinotecan (CPT-11) and its active metabolite SN-38 in the presence or absence of CM+. (Figure S2C). Therefore, altered PK of cisplatin or irinotecan could not account for the observed resistance.

To evaluate whether the systemic factors induce resistance to the tumor cells directly, we established an in vitro model in which we either performed cocultures of MSCs and tumor cells or treated the cultured tumor cells with chemotherapy in combination with the CM+ from the MSCs. We found that in vitro neither coculture with MSC nor incubation with CM+ could directly protect the tumor cells against chemotherapy, and no induction of resistance was observed (Figure 3E). This suggests the requirement of secondary secreted host factors. Indeed, we found that plasma, obtained from nontumor-bearing mice 1 hr after administration of the CM+, did induce resistance to cisplatin and irinotecan in our in vitro system (Figure 3F). We excluded the requirement of a potential cofactor from the plasma by adding the CM+ to plasma from untreated mice. This could not induce resistance (data not shown). In conclusion the induction of resistance to chemotherapy is not mediated via a direct effect of the systemic factors on the tumor cells but likely via the release of an intermediate factor(s) by the host tissue in the plasma that then directly prevents the induction of apoptosis in the tumor cells by chemotherapy.

MSC-Induced Resistance Is Mediated by the Release of Polyunsaturated Fatty Acids

In order to identify the resistance-inducing systemic factor(s) secreted by platinum-activated mouse MSCs, we followed a systematic metabolomics approach of stepwise CM fractionations (Figure 4A) and tested each fraction for its ability to induce chemoresistance in vivo. First, CM+ was separated into two

The graphs depict representative results from four individual experiments. Student's t test, all compared to chemotherapy alone: $^{**}p < 0.01$, $^{***}p < 0.001$. Data are expressed as mean ± SEM (n = 6 mice per group).
See also Figure S1.

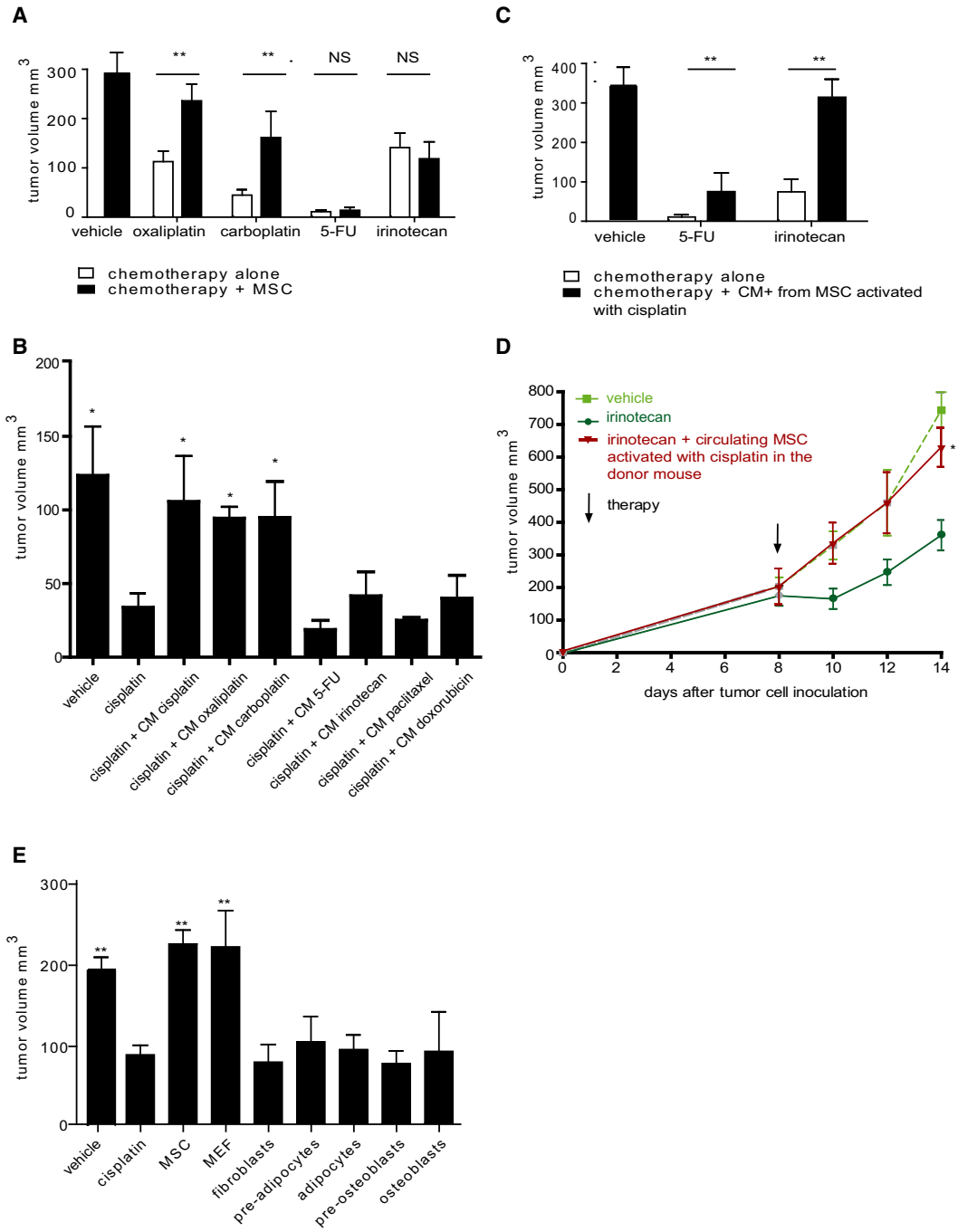

Figure 2. In Response to Platinum-Based Chemotherapy, MSCs Induce Resistance to Multiple Types of Chemotherapy

(A) Tumor volume of C26 cells 4 days after treatment with either oxaliplatin (10 mg/kg), carboplatin, irinotecan, and 5-FU (all 100 mg/kg) alone, or in combination with 100,000 MSCs i.v.

(B) Tumor volume of C26 cells 4 days after treatment with cisplatin alone or in combination with the CM from MSCs preincubated with different types of chemotherapy.

(C) Tumor volume of C26 cells 4 days after treatment with either 5-FU or irinotecan (both 100 mg/kg) alone or in combination with s.c. injections of CM+ from cultured MSCs preincubated with cisplatin (n = 3 mice per group).

(D) A total of 1000 circulating MSCs, harvested from the blood of tumor-bearing mice 30 min after treatment with cisplatin, was s.c. injected into tumor-bearing mice concomitantly with irinotecan treatment. Tumor growth of C26 cells either treated with vehicle control, irinotecan alone or irinotecan plus MSCs is plotted.

(E) Tumor volume 4 days after treatment with cisplatin in combination with s.c. injections of CM+ from the different cell types preincubated with cisplatin.

Data are expressed as mean ± SEM (n = 6 mice per group). The graphs depict representative results from three individual experiments. Student's t test, all compared to chemotherapy alone: nonsignificant (NS) $p > 0.05$, *$p < 0.05$, **$p < 0.01$.

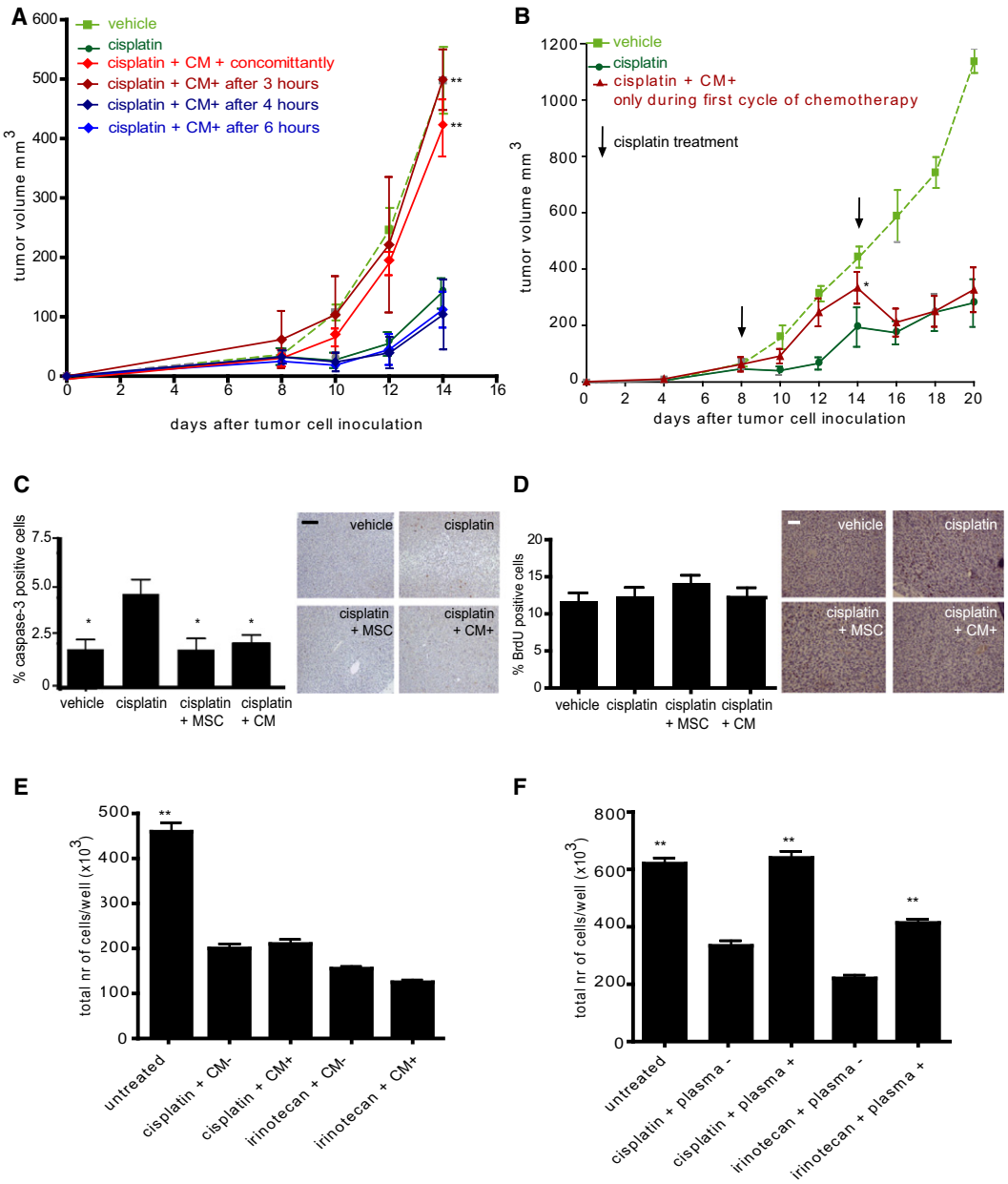

Figure 3. Induction of Resistance via an Indirect and Reversible Prevention of Apoptosis

(A) Tumor growth of C26 cells inoculated s.c. either untreated, treated with cisplatin alone or with cisplatin plus CM+ injected at different time points after administration of the chemotherapy.

(B) Tumor growth of C26 cells inoculated s.c. either untreated, treated with cisplatin alone or with cisplatin plus s.c. injections of the CM+ during the first cycle of chemotherapy. During the second cycle of chemotherapy, only cisplatin was administered in the latter two groups.

(C and D) Immunohistochemical analysis of tumors 4 days after start of treatment with cisplatin alone or combined with either MSC or CM+. Apoptosis was measured by staining for cleaved caspase-3 (C), proliferation was measured by BrdU (D), and the percentage of positive cells was determined for ten random fields per tumor. Scale bars, 50 μm.

(E) Number of C26 cells 24 hr after incubation in vitro with cisplatin (3 μM) with or without coadministration of CM+.

(F) Number of C26 cells 24 hr after incubation in vitro with cisplatin (3 μM) or irinotecan (250 μM) with or without coadministration of the plasma from mice 1 hr after administration of the CM+.

Data are expressed as mean ± SEM (n = 6 mice per group). The graphs depict representative results from three individual experiments. Student's t test all compared to chemotherapy alone: *$p < 0.05$, **$p < 0.01$.

See also Figure S2.

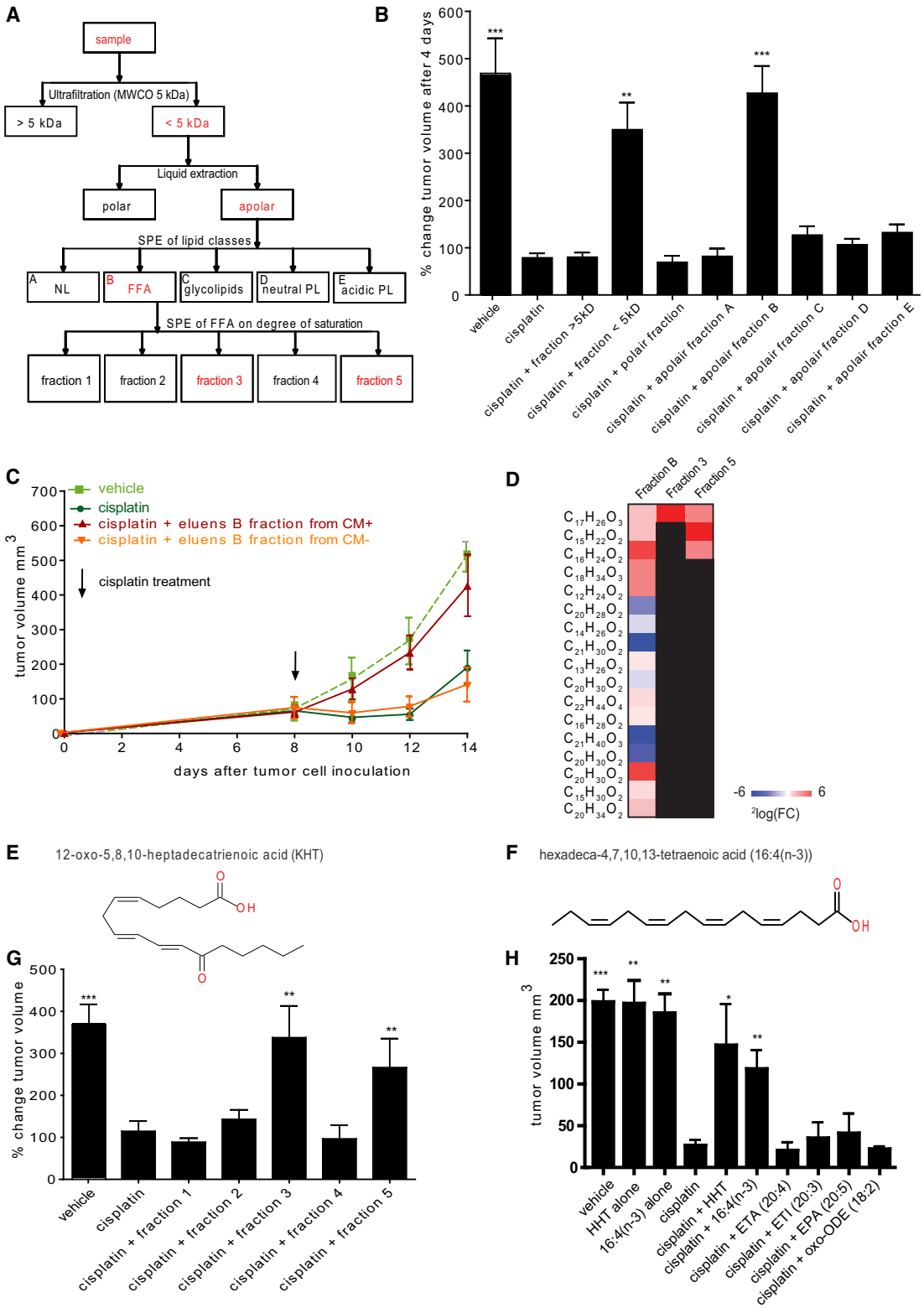

Figure 4. Systematic Fractionation of CM Reveals that the Secretion of Distinct Fatty Acids Induces Resistance

(A) CM from mouse MSCs was stepwise fractionated by size, liquid-liquid, and SPE resulting in a separation by lipid class. The FFAs were further fractionated based on degree of saturation. The ability of induction of resistance of each fraction was tested.

(B and G) Percent (%) change in tumor volume 4 days after start of therapy of C26 tumors, either untreated, treated with cisplatin alone or with one concomitant dose of the fractions of the CM+ or individual factors s.c. (n = 4 mice per group).

fractions based on size, using a 5 kDa cutoff to roughly distinguish between proteins and metabolites. Only the fraction containing the components smaller than 5 kDa induced resistance (Figure 4B). Liquid-liquid extraction was then performed that pointed to the presence of the active components in the apolar fraction (Figures 4A and 4B), suggesting the involvement of lipid-like factors. We next performed aminopropyl-Si solid phase extraction (SPE) to enrich for different lipid classes and found that only fraction B from CM+ retained the potential to induce resistance (Figures 4B and 4C). To characterize the differentially regulated CM metabolites in more detail, we subjected fraction B, enriched for free fatty acids (FFAs), to UPLC-Orbitrap mass spectrometry (MS), which enables accurate mass measurement and, thus, determination of the elemental composition of identified components. A highly complex mixture was observed (Figure S3A) including 69 factors with a FFA elemental composition (Table S1), 17 of which were differentially present in the CM+ compared to CM− (Figure 4D). To further reduce complexity, fraction B was separated by Ag-ion SPE based on the saturation level of the fatty acids. This resulted in two fractions that independently induced resistance in the mouse model (Figure 4G), suggesting that at least two factors are involved. MS experiments unambiguously identified the elemental composition of three masses: one differentially regulated FFA in fractions 3 and 5 with identical elemental composition, and two additional FFAs in fraction 5 (Figures 4D and S3B–S3F, and Table S2). The chromatographic properties in combination with the elemental composition and spectral fragmentation patterns by Orbitrap MS (data not shown) allowed us to propose that in fractions 3 and 5 the elemental composition $C_{17}H_{26}O_3$ belonged to 12-oxo-5,8,10-heptadecatrienoic acid (KHT), whereas in fraction 5, $C_{16}H_{24}O_2$ belonged to hexadeca-4,7,10,13-tetraenoic acid (16:4(n-3)) (Figures 4E and 4F). The identity of $C_{15}H_{22}O_2$ could not be unambiguously determined. To confirm our findings, we injected tumor-bearing mice with as little as 2 pmol of the KHT precursor HHT, which is readily converted to KHT (Agins et al., 1987), and 2.5 pmol of the purified 16:4(n-3) (Ishihara et al., 2000), and found that each of the PIFAs individually induced complete resistance to cisplatin (Figure 4H), whereas a range of other polyunsaturated fatty acids was inactive (Figure 4H and Table S3). Notably, the pure PIFAs did not enhance tumor growth without the administration of cisplatin (Figure 4H).

We were able to develop a sensitive MS assay only for 16:4(n-3) because we had access to the purified 16:4(n-3), whereas we unfortunately do not have purified KHT. Using this assay, we evaluated the kinetics of the release of 16:4(n-3) by the MSCs in response to chemotherapy. Incubation of the MSCs with 1 μM of the platinum compounds cisplatin, oxaliplatin, and carboplatin resulted in a 2.5-, 2.8-, and 2.8-fold increase of 16:4(n-3) relative to CM−. In line with the in vivo experiments, incubating the MSCs with the same concentrations of other chemotherapeutics did not, or to a lesser extent, lead to an increase in 16:4(n-3) i.e., 5-FU increased 0.77-fold, irinotecan 1.74-fold, doxorubicin 0.73-fold, and paclitaxel induced a 1.55-fold increase compared to the CM−. When we incubated MSCs with increasing concentrations of cisplatin, we found a dose-dependent increase of 16:4(n-3) in the CM (Figure S3G). This shows that the platinum-based chemotherapeutics are most powerful in inducing the release of 16:4(n-3), and that that effect is dose dependent.

MSC-Induced Resistance Is Prevented by Intervening with the Cyclooxygenase (COX)-1/Thromboxane Synthase (TXAS) Pathway

One of the identified PIFAs, KHT is known to be a by-product of thromboxane A2 synthesis. In response to various stimuli, often via an increase in intracellular calcium, phospholipases (PLA2 and PLC) become phosphorylated and, subsequently, release the large omega-3 and -6 fatty acids eicosapentaenoic acid (EPA) and arachidonic acid (AA) from the cell membrane. Intracellularly, these FFAs are further processed by the COX pathway leading to the production of various prostaglandins, leukotrienes, and via TXAS to thromboxane A2 (Agins et al., 1987; Naraba et al., 1998). All are rapidly acting and powerful lipids with broad systemic functions. To test whether intervening with this process could prevent resistance by the platinum-activated MSCs, we preincubated the cultured MSCs with various inhibitors targeting all the steps in the pathway, before the incubation of these MSCs with cisplatin. We used the phospholipase A2 inhibitors MAFP, DEDA, and the selective cPLA2 (calcium-dependent PLA2) inhibitor pyrrophenone, the phospholipase C inhibitors U73122 and D609, the intracellular calcium-chelating agent BAPTA AM, the calcium pump inhibitors verapamil and nicardipine, the COX inhibitor indomethacin, the selective COX-2 inhibitor celecoxib, the selective COX-1 inhibitor SC560, and the TXAS inhibitors ozagrel and furegrelate. Strikingly, we found that preincubation with inhibitors of PLA2, COX-1, and TXAS, or addition of an intracellular calcium chelator, blocked the induction of resistance by platinum-activated MSCs, whereas inhibition of PLC, the calcium pumps, or COX-2 had no effect (Figure 5A). Notably, in the CM of MSCs pretreated with either indomethacin or ozagrel, the PIFAs were significantly downregulated to levels lower than CM− (data not shown). When we added the PIFAs back to this CM and tested this CM in our mouse models, this fully restored the ability to induce resistance (Figure S4A). This suggests that the release of the two PIFAs by the MSCs in response to cisplatin is mediated by an intracellular calcium-mediated activation of PLA2, leading to the release of AA and EPA from the cell membrane that are the substrates for

(C) Tumor growth of C26 cells either untreated, treated with cisplatin alone or cisplatin plus one concomitant dose of the fatty acid fraction B from CM+ or CM− (s.c.).

(D) Differentially regulated fatty acids in the CM+/CM− fractions B, 3, and 5. The elemental composition as determined by high-accuracy FTMS is shown in a heat map. FC, fold change.

(E and F) Chemical structure of the identified PIFAs.

(H) Tumor volume 4 days after treatment with the PIFAs alone, cisplatin alone, or cisplatin plus one concomitant dose of either the identified PIFAs, KHT or 16:4(n-3), or similar fatty acids as controls (n = 6 mice per group).

The graphs depict representative results from two individual experiments. Data are expressed as mean ± SEM. Student's t test, all compared to chemotherapy alone: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

See also Figure S3 and Tables S1–S3.

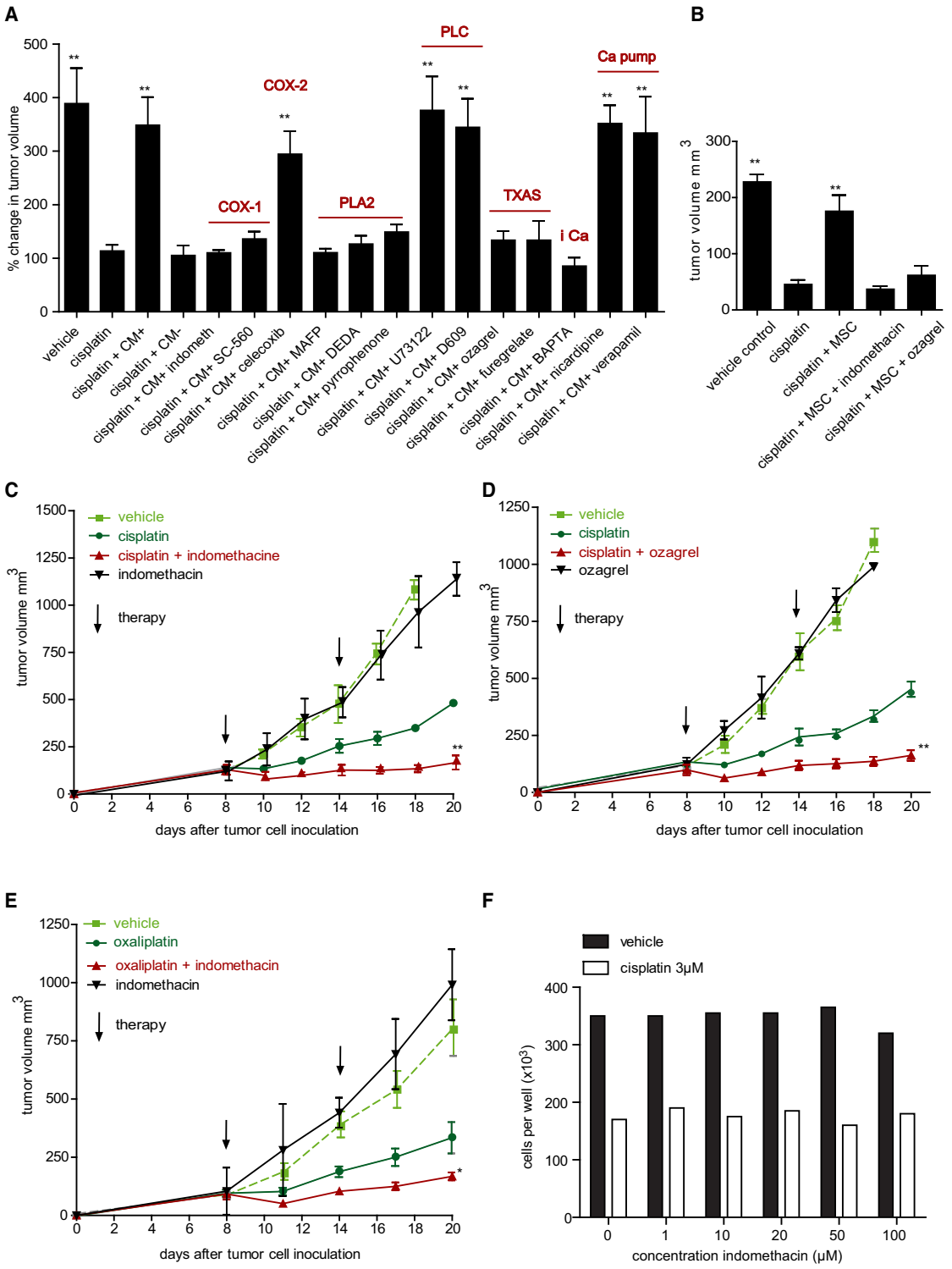

Figure 5. MSC-Induced Resistance Is Prevented by COX-1 Inhibition

(A) Tumor volume of C26 cells 4 days after start of treatment. Mice were either untreated, treated with cisplatin alone or with cisplatin plus s.c. injections of the CM+ from MSCs pretreated with the phospholipase A2 inhibitors MAFP, DEDA, and the selective cPLA2 inhibitor pyrrophenone, the phospholipase C inhibitors U73122 and D609, the intracellular calcium-chelating agent BAPTA AM, the calcium pump inhibitors verapamil and nicardipine, the COX inhibitor indomethacin, the selective COX-2 inhibitor celecoxib, the selective COX-1 inhibitor SC560, and the TXAS inhibitors ozagrel and furegrelate (n = 4 mice per group).

(B) Tumor growth of C26 cells either untreated, treated with cisplatin alone or with cisplatin plus MSC with or without pretreatment with indomethacin (2 mg/kg) or ozagrel (20 mg/kg) s.c. 1 day and 1 hr before cisplatin (n = 5 mice per group).

further conversion by COX and TXAS. In line with these findings, microarray data, comparing untreated MSCs with cisplatin-treated MSCs, showed no relevant differences in gene expression (data not shown). Furthermore, the prior observation that PIFAs are already released from MSCs almost immediately after exposure to cisplatin, within 1.5 hr, also suggests that transcriptional regulation is less likely. In addition, as shown by western blots, the level of COX remained unchanged in the MSCs after treatment with cisplatin (data not shown). Together, this suggests that the increased production of the PIFAs is due to an increase in the availability of the substrates. Thus far, 16:4(n-3) has only been described to be formed via peroxisomal oxidation from EPA (Williard et al., 1998). Given our results, it is likely that similar to KHT, 16:4(n-3) functions downstream of the COX-TXAS pathway.

We confirmed these findings in vivo and found that, indeed, in our mice models in which we administrated the MSCs i.v., addition of only two doses of indomethacin (2 mg/kg) or ozagrel (20 mg/kg) just before the chemotherapy completely prevented the MSC-induced resistance (Figure 5B). The role of MSC-mediated fatty acid production in chemoresistance was further supported by the observation that in both C26 tumors and LLC tumors, without the administration of additional MSCs, indomethacin and ozagrel significantly enhanced the antitumor efficacy of cisplatin and oxaliplatin alone in vivo (Figures 5C–5E; Figures S4B and S4C). Interestingly, neither indomethacin nor ozagrel alone had any antitumor effect when administered on this dosing schedule. Notably, indomethacin did not enhance the efficacy of cisplatin in vitro (Figure 5F), which could suggest that addition of indomethacin or ozagrel enhances the antitumor efficacy of cisplatin by preventing the platinum-induced activation and subsequent PIFA release by the endogenous MSCs in vivo. This is supported by the fact that indomethacin did not enhance the antitumor efficacy of irinotecan in vivo (Figure S4D).

In conclusion these experiments indicate that specifically TXAS and COX-1, but not COX-2, inhibition prevented MSC-induced resistance and enhanced chemotherapy efficacy. Therefore, these results support the notion that inhibition of COX-1/TXAS pathways could provide a drugable target to prevent PIFA-induced chemoresistance.

Evidence for the Clinical Relevance of MSC-Induced Resistance to Chemotherapy

To validate our findings in cancer patients, we first measured the levels of MSCs in whole blood of a group of 50 cancer patients with different types of tumors. We found a significant increase in MSC levels in the peripheral blood of cancer patients with metastatic disease compared to cancer patients after radical resection of the tumor with no evidence of residual disease (Figure S5A). These findings indicate that MSCs are present in the circulation and, therefore, will be exposed to chemotherapy. Furthermore, the number of circulating MSCs is comparable to the number of MSCs sufficient to confer chemoresistance in mouse models. To demonstrate the clinical relevance of our findings, we isolated MSCs from the BM of three healthy human volunteers. MSCs from each donor were cultured and characterized by FACS analysis, the number of cfu-F was determined, and MSC phenotype was confirmed by multilineage differentiation potential into chondrocytes, adipocytes, and osteoblasts (Figures S5B and S5C). The results of these extensive characterizations were previously published (Prins et al., 2009; Siddappa et al., 2008). MSCs from each subject were injected i.v. in nude mice bearing s.c. growing human MDA-MB-231 breast cancer cells. Each human MSC isolate induced a similar cisplatin resistance as previously observed for mouse MSCs. Furthermore, treatment of these mice with weekly cisplatin for 5 weeks showed that the protective effect of the MSCs, administered only before the first dosage of cisplatin, persisted during the following cycles of chemotherapy (Figures 6A and 6B). From these experiments we concluded that both mouse and human MSCs can induce resistance in response to cisplatin treatment when added to the circulation of tumor-bearing mice. Furthermore, mice treated with daily s.c. injections of CM harvested from cisplatin-preincubated cultures of human MSCs (CM+) became completely resistant to cisplatin. CM from untreated human MSCs (CM−) had no effect on the in vivo response to cisplatin (Figure 6C). Through MS we confirmed the presence of 16:4(n-3) and KHT in the CM+ of activated human MSCs similar to PIFAs secreted by mouse MSCs (Figure S5D). Finally, we measured the levels of 16:4(n-3) in plasma samples from ten esophageal cancer patients treated with cisplatin-based chemotherapy and ten breast cancer patients treated with nonplatinum-based chemotherapy. In an exploratory analysis we found a significant increase in 16:4(n-3) hours after treatment with cisplatin-based chemotherapy compared to the patients treated with nonplatinum-based chemotherapy ($p = 0.04$) (Figure 6D). Overall, based on the preclinical data and exploratory clinical data, these results indicate that our identified mechanism of resistance by MSCs via the release of PIFAs can be translated to the clinic.

Fatty acids are also present in various food products and supplements. We found that our identified PIFA 16:4(n-3) is abundantly present in commercially available fish oil products (0.4–0.6μM 16:4(n-3)) and algae extracts (27μM 16:4(n-3)). Fish oil products are frequently used by cancer patients because of their perceived positive health effects, such as preventing cachexia and cardiovascular events, anti-inflammatory properties, prevention of tumor growth, and reduction of chemotherapy-induced side effects (Berquin et al., 2008; Jiang et al.,

(C–E) Tumor growth of C26 cells untreated, treated with cisplatin (C and D) or oxaliplatin (E) alone, indomethacin (C and E) or ozagrel (D) alone, or with cisplatin and indomethacin (C), cisplatin and ozagrel (D), or oxaliplatin and indomethacin (E). Both indomethacin and ozagrel were administered s.c. 1 day and 1 hr before cisplatin or vehicle control at days 8 and 14 after tumor cell inoculation (n = 8 mice per group). The graphs depict representative results from three individual experiments.

(F) In vitro cytotoxicity of cisplatin (3 μM), indomethacin, or cisplatin plus indomethacin. Indomethacin was administered at doses ranging from 1 to 100 μM. Viable cells were counted 24 and 48 hr after start of therapy.

Data are expressed as mean ± SEM. Student's t test, all compared to chemotherapy alone: *$p < 0.05$, **$p < 0.01$.

See also Figure S4.

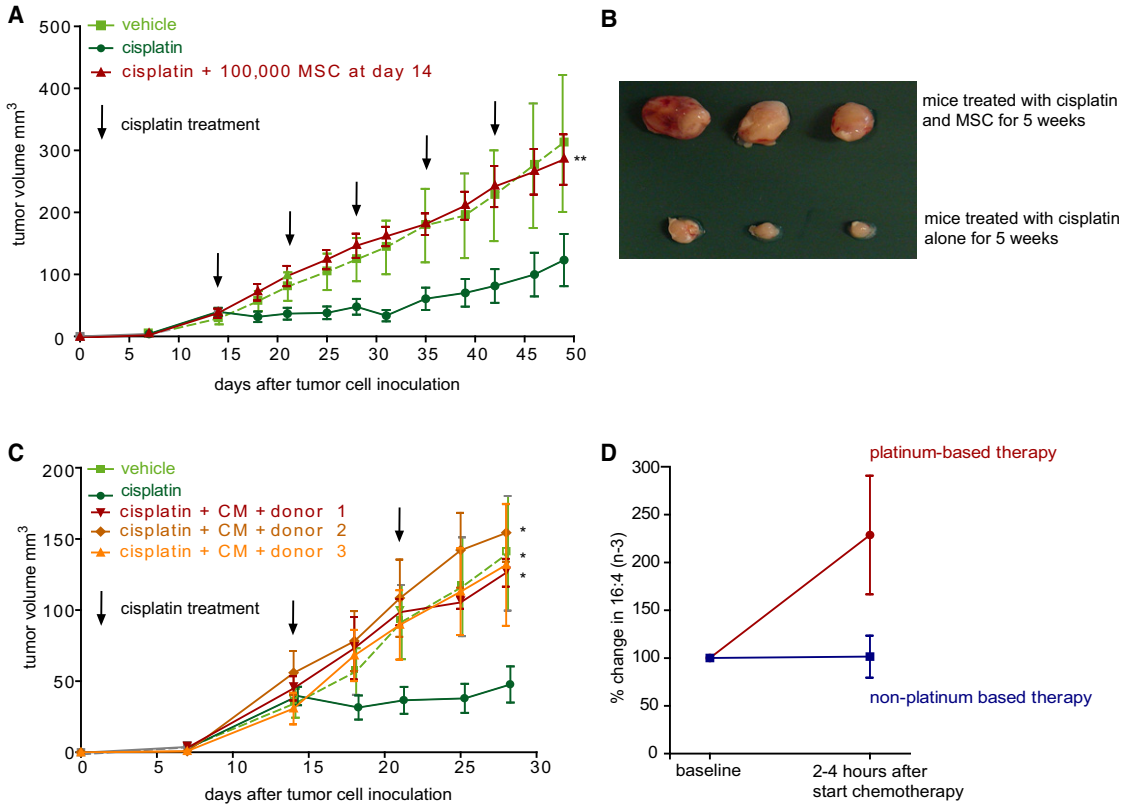

Figure 6. Human MSCs Secrete the Same PIFAs that Induce Resistance in a Breast Cancer Xenograft Model

(A and B) Tumor growth of MDA-MB-231 cells treated with either vehicle control, cisplatin or with cisplatin plus 100,000 i.v.-injected human MSCs concurrently with only the first administration of the cisplatin (6 mg/kg). Cisplatin therapy was repeated weekly (arrows) (n = 5 mice per group).

(C) Tumor growth of MDA-MB-231 cells either untreated, treated with cisplatin alone weekly or with cisplatin weekly plus 100 μl CM+ (s.c.) from human MSCs derived from three healthy individuals (n = 5 mice per group). The graphs depict representative results from two individual experiments.

(D) 16:4(n-3) levels were measured using MS in plasma samples from esophageal cancer patients (n = 10) treated with cisplatin-based therapy compared to breast cancer patients (n = 10) treated with nonplatinum-based therapy. Percent (%) change is shown between baseline and 2–4 hr after chemotherapy (p = 0.04). Data are expressed as mean ± SEM. Student's t test, compared to chemotherapy alone unless otherwise indicated: *p < 0.05, **p < 0.01.

See also Figure S5.

2010; Shaikh et al., 2010; van der Meij et al., 2010). We hypothesized that the use of fish oil containing our identified fatty acids may have an adverse effect on the antitumor effects of chemotherapy. To test this, we fed tumor-bearing mice either purified PIFAs or commercially available fish oil products and treated them with cisplatin. We found that orally administered, pure PIFAs induced resistance to cisplatin in our tumor models (Figure 7A). Furthermore, a single oral dose of 100 μl of either two different commercially available fish oil products or algae extracts resulted in a neutralization of the antitumor effects of cisplatin in both C26 and LLC tumors (Figure 7B; Figure S6A). Administration of only fish oils or algae did not alter tumor growth (Figure S6B). Orally administered EPA, the main component of most fish oil products, that served as a control in both tumor models had no effect. Importantly, both fish oil products and the algae extract induced a complete resistance to chemotherapy at doses similar to the advised daily dose in humans. These results provide additional support for the clinical relevance of these fatty acids in the development of resistance to chemotherapy.

DISCUSSION

Our study reveals an important mechanism of resistance to chemotherapy mediated by MSCs. We show that MSCs, activated by platinum-based chemotherapy, secrete unique fatty acids that, in minute quantities, confer resistance to multiple types of chemotherapy. We identified two distinct PIFAs, namely KHT and 16:4(n-3), responsible for the induction of resistance by the MSCs. We tested the purified PIFAs in our mouse models and confirmed that they are extremely potent in inducing resistance to chemotherapy. In contrast to other related polyunsaturated fatty acids had no effect.

We provide several lines of evidence that point to the clinical importance of platinum-based activation of MSCs in relation to therapy response. First, MSCs are recruited to the circulation of cancer patients with a tumor in situ. Second, in different tumor-bearing mouse models, circulating MSCs were activated by platinum-based chemotherapy in vivo and, subsequently, induced resistance to chemotherapy. These findings indicate that circulating MSCs will be exposed to chemotherapy at the

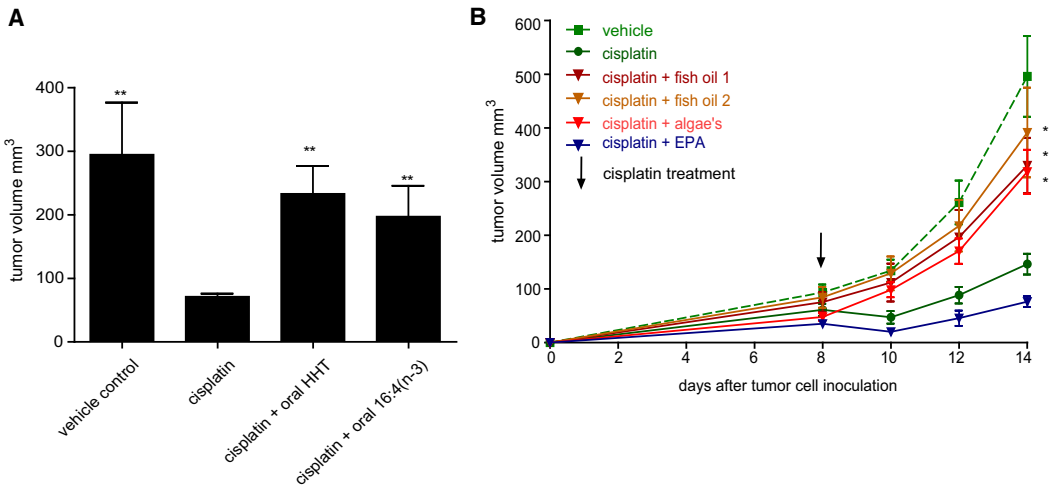

Figure 7. Algae Extracts and Commercially Available Fish Oil Products Induce Resistance to Chemotherapy

(A) Tumor volume of C26 cells 4 days after treatment with either vehicle control, cisplatin (6 mg/kg) or cisplatin plus oral coadministration of HHT and 16:4(n-3), respectively.

(B) Tumor growth of C26 cells either untreated or treated with cisplatin alone or with cisplatin plus orally administered fish oil products, algae extracts, or EPA, the main component of most fish oil products.

Data are expressed as mean ± SEM (n = 6 mice per group). The graphs depict representative results from two individual experiments. Student's t test, all compared to chemotherapy alone: *$p < 0.05$, **$p < 0.01$.

See also Figure S7.

start of treatment and that the number of circulating MSCs in cancer patients is comparable to the number of MSCs sufficient to confer chemoresistance in mouse models. Third, human and murine MSCs secrete the specific PIFAs KHT and 16:4(n-3) upon stimulation with platinum drugs, and these PIFAs can indeed induce resistance in a human xenograft model. Fourth, we found a significant increase in 16:4(n-3) hours after cisplatin-based chemotherapy in patients compared to patients treated with nonplatinum-based chemotherapy. Finally, it is commonly observed that resistance to chemotherapy in patients develops at all sites simultaneously, which, albeit speculative, could support the presence of systemic factors such as the PIFAs described here being responsible for the induction of resistance.

Platinum-based chemotherapy is the mainstay of care for multiple types of cancer, including colorectal, ovarian, esophageal, testicular, lung, and head and neck cancer (Kelland, 2007). Therefore, preventing the development of resistance in these patients will be beneficial for a large group of patients. Interventions directed against either MSCs or the identified PIFAs may provide opportunities to enhance the efficacy of chemotherapy and prevent the development of resistance. Very little is known about the identified PIFAs belonging to the omega-3 and oxo class of fatty acids, respectively. In general, omega-3 and oxo fatty acids, are biologically active lipids with specific receptors in both the cell membrane and nucleus. (Wang and Dubois, 2010; Okuno et al., 2008). For KHT and 16:4(n-3) no receptors or downstream signaling pathways have been described. We show that the PIFAs do not induce resistance directly but induce the release of an intermediate factor(s) by the host tissue in the plasma, which then directly prevents the induction of apoptosis of tumor cells by chemotherapy.

By considering the mechanism of normal production of eicosanoids, we have been able to identify strategies to interfere with the production of the identified PIFAs, and our results indicate that this is a highly effective method to prevent resistance to chemotherapy. We show that inhibition of intracellular calcium, PLA2, TXAS, and COX-1, but not COX-2 inhibition, prevents the release of PIFAs by MSCs. We show that the use of a COX-1 inhibitor or a TXAS inhibitor in combination with platinum-based chemotherapy significantly enhances the chemotherapy efficacy. In line with the somewhat disappointing outcome of multiple clinical trials that have been performed using COX-2 inhibitors in combination with chemotherapy (Köhne et al., 2008; Pierga et al., 2010), our findings suggest that inhibition of COX-1, TXAS, or other enzymes leading to the production of these resistance-inducing PIFAs may be more effective.

Finally, our findings may also have important implications for the current use of MSCs to promote tissue repair in clinical trials for cancer-unrelated diseases such as immune disorders and cardiovascular diseases (Psaltis et al., 2008; Djouad et al., 2009). Our study demonstrates that MSCs can be specifically activated to secrete cytoprotective factors. This finding may be utilized in the design of clinical trials in which MSCs are used in various nononcological diseases. Additionally, our findings may have implications for the use of commercially available fish oil products and algae by cancer patients during therapy. We show that orally administered fish oil products in doses similar to the converted advised daily dose in humans already induce resistance to chemotherapy in mice. Therefore, the use of these products during chemotherapy treatment should be avoided.

In summary our study identifies a systemic mechanism of resistance via activation of MSCs by platinum-based chemotherapy with the subsequent release of two specific resistance-inducing PIFAs. Our findings introduce important players to the field of chemotherapy resistance and indicate that the

TXAS and COX-1 pathway may be drugable pathways to prevent PIFA-induced chemoresistance.

EXPERIMENTAL PROCEDURES

MSC Isolation and Culture

Murine MSCs were isolated from the BM or blood from syngenic mice using immunomagnetic selection and primary cell culture. Human MSCs were isolated from BM aspirates from healthy donors and cultured as previously described (Prins et al., 2009). This was approved by the Institutional Ethical Review Board of the University Medical Center (UMC) Utrecht, and written informed consent was obtained from all donors. For identification, BM from GFP+ mice (C57BL/6-Tg(UBC-GFP)30Scha/J mice; The Jackson Laboratory) was used. MSC phenotype of the cultured MSCs was confirmed by FACS analysis and multilineage differentiation.

Mouse Experiments

All animal procedures were approved by the UMC Utrecht Animal Care Ethics Committee and are in agreement with current Dutch Law on Animal Experiments. C26 colon carcinoma cells (1×10^6 cells), LLC cells (0.5×10^6 cells), and MDA-MB-231 cells (3×10^6 cells) were s.c. implanted into BALB/c, C57Bl/6, or athymic nude mice. Tumor size was assessed by caliper measurements; volume was calculated (($L \times W^2$) $\times$ 0.5). Chemotherapy was administered intraperitoneally (i.p.), weekly at MTD level (cisplatin 6 mg/kg, oxaliplatin 10 mg/kg, carboplatin, 5-FU, and irinotecan at 100 mg/kg). Control mice received appropriate vehicles. MSCs (both cultured or freshly isolated from the BM) were administered either i.v. or s.c. at the start of chemotherapy treatment. CM was harvested from the different cell types after 1–24 hr, from both untreated cells (CM−) and cells pretreated with different types of chemotherapy for 30 min to 4 hr (CM+) a 100 μl of CM, or fractions was administered s.c. KHT precursor 12-HHT (Agins et al., 1987), 16:4(n-3) (Ishihara et al., 2000), and control fatty acids were tested as described in Table S3. Furthermore, two commercially available fish oil products and homogenized extracts from Ulva Pertussa algae were tested. The inhibitors MAFP (100 μM), DEDA (100 μM), pyrrophenone (1 μM), U73122 (5 μM), D609 (100 μM), BAPTA AM (1 μM), verapamil (10 μM), nicardipine (10 μM), indomethacin (10 and 100 μM), celecoxib (10 and 100 μM), SC560 (1 mM), ozagrel (1 mM), and furegrelate (1 mM) were tested in vitro; indomethacin (2 mg/kg, s.c.) and ozagrel (20 mg/kg, s.c.) were also tested in vivo for their ability to interfere with the induction of resistance by MSCs.

Fractionation and MS Analysis

CM was systematically fractionated as depicted in Figure 4A; each fraction was assessed for the ability to induce resistance in vivo. Fractions from CM+ were compared to CM− by ultra-high pressure liquid chromatography coupled to Orbitrap mass spectrometer; differentially secreted substances were analyzed with 5 ppm mass tolerance and subjected to database searches using Nature Lipidomics Gateway (www.lipidmaps.org) and tested individually.

In Vitro Experiments

C26 cells were treated in vitro with cisplatin (3 μM) or irinotecan (150 μM) alone or combined with either CM+/CM− or with SFM supplemented with 4% plasma harvested from nontumor-bearing mice 1 hr after treatment with either CM+ or CM−. Cell survival for all experiments was determined by counting the number of viable cells, using trypan blue exclusion, 24 and 48 hr after start therapy.

Patient Samples

Blood samples were collected from 43 cancer patients before receiving chemotherapy at the UMC Utrecht, The Netherlands. The study was approved by the Institutional Ethical Review Board of the UMC Utrecht, and written informed consent was obtained from all patients. Blood was collected in a Cell Preparation Tube. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated. Plasma was stored immediately at −80°C. The PBMCs were washed once in RPMI and stored in 10% DMSO at −80°C until analysis. The number of MSCs in the PBMC fraction was quantified by flow cytometry. MSCs were defined as CD45−, CD90+, CD73+, and CD105+, and calculated to the number of cells per milliliter of blood using the mononuclear cell count. The levels of 16:4(n-3) were determined in the citrate plasma samples of ten esophageal cancer patients and ten breast cancer patients.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.ccr.2011.08.010.

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