

Microenvironment mesenchymal cells protect ovarian cancer cell lines from apoptosis by inhibiting XIAP inactivation

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Epithelial ovarian carcinoma is characterized by high frequency of recurrence (70% of patients) and carboplatin resistance acquisition. Carcinoma-associated mesenchymal stem cells (CA-MSC) have been shown to induce ovarian cancer chemoresistance through trogocytosis. Here we examined CA-MSC properties to protect ovarian cancer cells from carboplatin-induced apoptosis. Apoptosis was determined by Propidium Iodide and Annexin-V-FITC labelling and poly-ADP-ribose polymerase cleavage analysis. We showed a significant increase of inhibitory concentration 50 and a 30% decrease of carboplatin-induced apoptosis in ovarian cancer cells incubated in the presence of CA-MSC-conditioned medium (CM). A molecular analysis of apoptosis signalling pathway in response to carboplatin revealed that the presence of CA-MSC CM induced a 30% decrease of effector caspases-3 and -7 activation and proteolysis activity. CA-MSC secretions promoted Akt and X-linked inhibitor of apoptosis protein (XIAP; caspase inhibitor from inhibitor of apoptosis protein (IAP) family) phosphorylation. XIAP depletion by siRNA strategy permitted to restore apoptosis in ovarian cancer cells stimulated by CA-MSC CM. The factors secreted by CA-MSC are able to confer chemoresistance to carboplatin in ovarian cancer cells through the inhibition of effector caspases activation and apoptosis blockade. Activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway and the phosphorylation of its downstream target XIAP underlined the implication of this signalling pathway in ovarian cancer chemoresistance. This study reveals the potentialities of targeting XIAP in ovarian cancer therapy.

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Subject Category: Cancer

Ovarian cancer is the most lethal gynaecological cancer due to late diagnosis and high level of recurrence (70% of patients). Patients initially respond to standard treatments that is a cytoreductive surgery and subsequent platinum-based chemotherapy. However, recurrence is characterized by chemoresistance leading to 5-year survival < 30%.^{1,2} More and more evidence pointed the involvement of non-tumoural cells in chemoresistance acquisition. Indeed, this microenvironment has become recognized as a major factor influencing the growth of cancer and impacting the outcome of therapy. Although the niche cells are not malignant *per se*, their role in supporting cancer growth is so vital for the survival of the tumour that they have become an attractive target for chemotherapeutic agents.³ Meads *et al.*⁴ have shown that environment-mediated drug resistance is rapidly induced by signalling events from the tumour microenvironment and is likely to be reversible because removal of the microenvironment restores the drug sensitivity.

Inside the microenvironment, we focused on the potential role of mesenchymal stem cells (MSCs; bone marrow-derived

MSCs (BM-MSC) or carcinoma-associated MSCs (CA-MSC) on the chemoresistance acquisition. MSCs are multipotent cells capable of differentiating into numerous cell types including adipocytes, osteoblasts, chondrocytes, fibroblasts, perivascular and vascular structures.⁵ MSCs are recruited in large numbers to the stroma of developing tumours as they constantly produce paracrine and endocrine signals mobilizing it from the bone marrow.⁶ They are defined as CA-MSCs and present some characteristic markers of carcinoma-associated fibroblasts (expression of PDGFR, FAP, etc.).⁷ Such MSCs are found to stimulate tumour growth, enhance angiogenesis and promote metastasis formation through the release of a large spectrum of growth factors and cytokines.^{6,8,9} Little is known on the mechanism leading to the transformation of a BM-MSC to a CA-MSC.^{10,11} Roodhart *et al.*,⁶ Xu *et al.*,¹² Hao *et al.*,¹³ Jin *et al.*¹⁴ and our group,¹⁵ have recently shown that MSCs are involved in the development of chemoresistance to multiple types of chemotherapies.

Among the several mechanisms involved in drug resistance, efflux pumps such as ABC proteins have an important

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Abbreviations: BM-MSC, bone marrow-derived mesenchymal stem cells; CA-MSC, carcinoma-associated mesenchymal stem cells; CM, conditioned medium; FasL, Fas ligand; FLIP, FLICE-like inhibitory protein; HOAC, human ovarian adenocarcinoma cells; IAP, inhibitor of apoptosis protein; IC50, inhibitory concentration 50; IL-6, interleukin-6; IL-8, interleukin-8; MSC, mesenchymal stem cells; PARP, poly-ADP-ribose polymerase; PI3K, phosphatidylinositol 3-kinase; S1P, sphingosine 1-phosphate; XIAP, X-linked inhibitor of apoptosis protein

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role in the uptake and distribution of therapeutic drugs, and their expression in the target tissue has been associated with resistance to treatment.¹⁶ Rafii *et al.*¹⁷ have shown that CA-MSC (called Hospicells in their study) are able to confer chemoresistance to ovarian and breast cancer cells by direct cell–cell contact and exchange of membrane patches (oncologic trogocytosis) and notably efflux pumps.

A recent study showed the protective effect and cell–cell dependency of MSC on drug-induced apoptosis in leukaemia cells.¹⁸ Other studies indicated a role of MSC-secreted factors in chemoresistance. MSC could induce chemoresistance through the release of multiple factors such as polyunsaturated fatty acids, interleukin-6 (IL-6) and vascular endothelial growth factor in the neighbourhood of tumours cells (for review see Castells *et al.*¹⁹).

Chemoresistance apparition can result from the inability of the cells to undergo apoptosis in response to chemotherapeutic agent caused by intracellular survival factors.²⁰ Apoptosis is a well-regulated mechanism controlled by pro- and anti-apoptotic proteins and triggered by extracellular or intracellular signals such as tumour necrosis factor α , Fas ligand (FasL), DNA-damaging agents or mitochondria activation. The activation of caspases is essential for propagating the apoptotic signal and for cellular protein degradation. The initiator caspases (caspase-8 and -10) are first activated upon extracellular stimuli, whereas the caspase-9 is activated by the mitochondrial pathway. Once activated by autoproteolytic cleavage, the initiator caspases cleave and activate the effector caspases (caspase-3, -6 and -7), which are responsible for the degradation of essential cellular substrates such as PARP (poly-ADP-ribose polymerase). (For review see Chipuk and Green²¹).

The activation of caspases represents an aetiological factor in the resistance of cancer cells to cytotoxic agents.²² Indeed potent caspase inhibitors such as IAP (inhibitor of apoptosis protein) family proteins or FLIP (FLICE-like inhibitory protein), and also the anti-apoptotic phosphatidylinositol 3-kinase (PI3K)/Akt signalling are involved in chemoresistance of ovarian cancers (for review see Fraser *et al.*²³). Platinum salts, reference treatment for ovarian cancer, activate DNA damage response triggering intrinsic and extrinsic apoptosis. For example, cisplatin has been shown to upregulate the expression of a number of apoptosis inducers including p53,²⁴ Fas and FasL,²⁵ and to downregulate anti-apoptotic proteins such as protein kinase B/Akt²⁶ or XIAP (X-linked IAP).²⁷ XIAP also known as inhibitor of apoptosis inhibitor 3 or baculoviral IAP repeat-containing protein 4 is an inhibitor of caspases that belongs to the IAPs family protein.²⁸ High expression of XIAP has been noticed in ovarian cancer cells and could be involved in cisplatin chemoresistance. Indeed overexpression of XIAP or Akt rendered chemosensitive ovarian cancer cells resistant to cisplatin, and downregulation of these proteins in chemoresistant counterparts by antisense or dominant-negative expression facilitated cisplatin-induced apoptosis.^{24,26,27}

In the present study, we examined the possible role of conditioned medium (CM) of CA-MSC or BM-MSC in carboplatin resistance in chemosensitive and chemoresistant ovarian adenocarcinoma cells. We showed that this CM contains factors that protect adenocarcinoma cells from carboplatin-induced apoptosis. CA-MSC secretions inhibited

the activity of effector caspases and induced activation of the PI3K/Akt pathway signalling and the phosphorylation of the downstream target XIAP.

Results

CM from CA-MSC as well as BM-MSC confer chemoresistance to ovarian cancer cells. We previously showed that the recruitment of CA-MSC (Hospicells) is proportional to the chemoresistance status of the tumours.¹⁷ We evaluated here if the factors released by CA-MSC could be associated to chemoresistance in ovarian cancer cells.

Platinum salts are known to induce cell growth inhibition of ovarian cancer cells.²⁹ We analysed if carboplatin-mediated growth inhibition of ovarian cancer cells could be modified by secreted factors from MSC. First, we determined the drug concentrations to inhibit 50% cell growth (inhibitory concentration 50, IC₅₀) in response to carboplatin in three different human ovarian adenocarcinoma cells (HOACs; OVCAR-3, IGROV-1 and SKOV-3 cells), as well as in CA-MSC and BM-MSC. Both CA-MSC and BM-MSC were highly sensitive to carboplatin treatment with a dose-dependent decrease of cell viability (IC₅₀ = 40.5 ± 12.5 μ M for CA-MSC and 40 ± 10 μ M for BM-MSC) (Figure 1d and Supplementary Figure 1a). OVCAR-3 and IGROV-1 cells were also chemosensitive but with higher IC₅₀ (IC₅₀ = 136 ± 20 and 145 ± 25 μ M, respectively). SKOV-3 cells were chemoresistant with a maximum of about 50% inhibition of cell growth and with an IC₅₀ of 350 ± 18 μ M (Figures 1a–c). Incubation of ovarian cancer cells in the presence of CA-MSC or BM-MSC CM during 24 h before treatment with carboplatin for 48 h significantly increased cell viability and IC₅₀ in OVCAR-3 and IGROV-1 cells (IC₅₀ = 245 ± 17 and 235 ± 26 μ M, respectively, for CA-MSC CM and IC₅₀ = 262.25 and 250.38 μ M, respectively, for BM-MSC CM). We also observed an increase of IC₅₀ in SKOV-3 cells cultured in the presence of MSCs CM (IC₅₀ = 350 ± 18 μ M without CM and IC₅₀ = 601 ± 48 and 611 ± 32 μ M with CA-MSC and BM-MSC CM, respectively), but the overall cell viability was not significantly affected (Figures 1a–c and Supplementary Figure 1a). Notably, we also observed taxane chemoresistance acquisition through MSCs CM in the three ovarian cancer cells lines but with a lesser extend (data not shown).

These results indicated that both CA-MSC and BM-MSC enhance ovarian cancer cells chemoresistance through the molecules they secrete.

CA-MSC and BM-MSC (secretions) protect ovarian cancer cells from carboplatin-induced apoptosis. Apoptosis deregulation is a key factor in the development of chemoresistance, and in particular molecular mechanism of cisplatin resistance involved apoptosis dysregulation.³⁰ To test the role of CA-MSC and BM-MSC secretions in the protection of ovarian cancer cells from carboplatin-induced apoptosis, we evaluated the proportion of apoptotic cells in response to carboplatin by analysing phosphatidyl serine externalization and membrane permeability by flow cytometry after Annexin-V-FITC/propidium iodide labelling and PARP cleavage by western blot. Incubation of chemosensitive cells, that is, OVCAR-3 cells, with carboplatin for 48 h induced

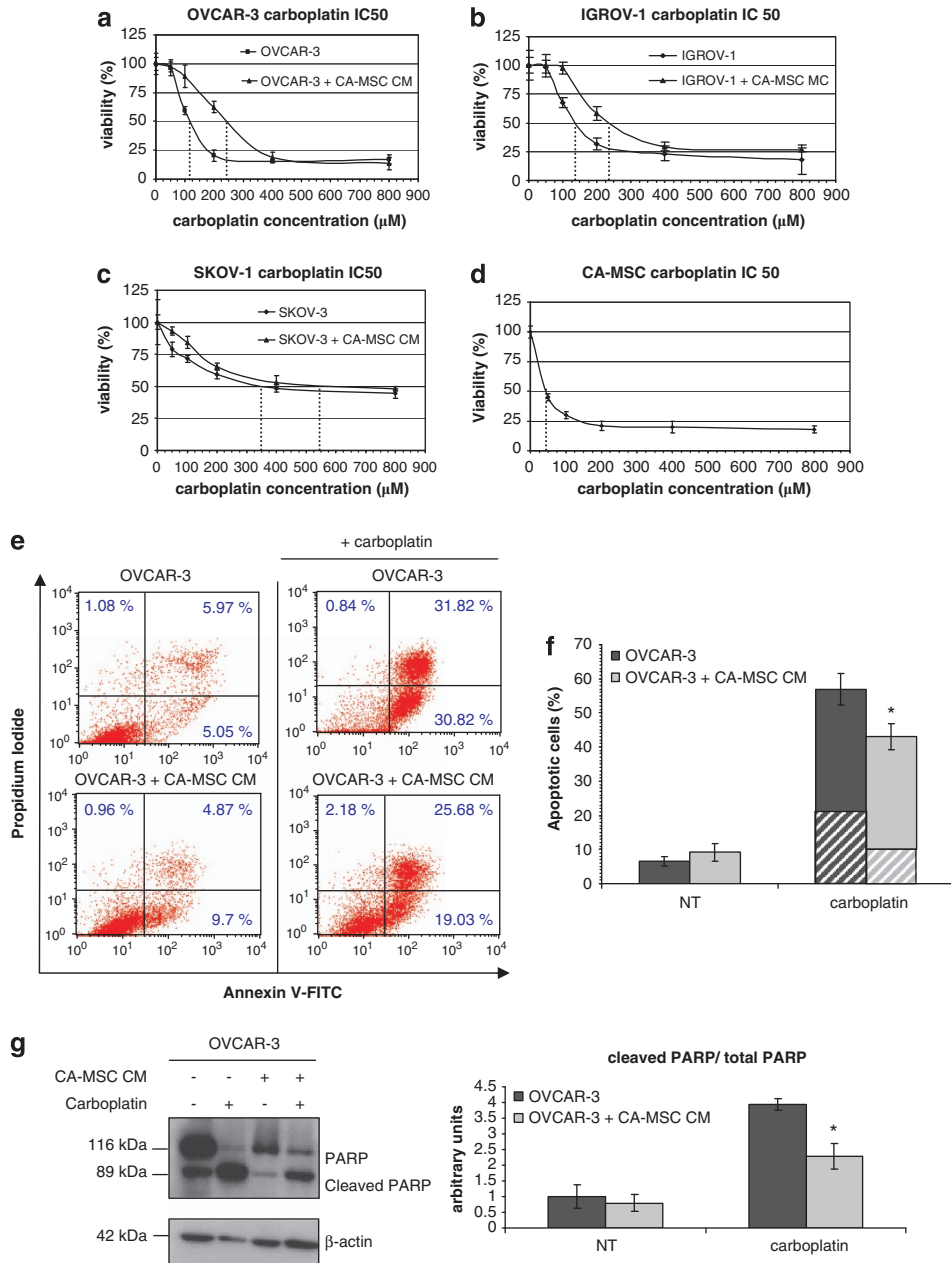


Figure 1 CA-MSC protect ovarian cancer cells from carboplatin-induced growth inhibition. Ovarian cancer cells cultured alone or in the presence of CA-MSC CM were treated with increasing concentrations of carboplatin for 48 h. Cell viability was reported for OVCAR-3 (a), IGROV-1 (b), SKOV-3 (c) and CA-MSC (d). Dotted line corresponded to 50% viability. OVCAR-3 cultured alone or in the presence of CA-MSC CM were treated with (+) or without (-) or (NT) 250 μM carboplatin for 48 h. (e and f) Apoptosis was measured by flow cytometry analysis after propidium iodide and Annexin-V-FITC labelling. Flow cytometry dot plot from a representative experiment (e). Histogram representing the apoptotic cells population (Annexin-V-positive cells + Annexin-V/propidium iodide-positive cells) for each condition (f). Hatched histogram represent Annexin-V-positive cells only (mean \pm S.E.M.; $n = 3$). * $P < 0.05$ versus OVCAR-3 + carboplatin. (g) PARP cleavage was analysed by western blot. Cleaved PARP level was normalized to total PARP (mean \pm S.E.M.; $n = 3$). * $P < 0.05$ versus OVCAR-3 + carboplatin

57 \pm 5% of apoptosis evaluated by the proportion of Annexin-V-FITC and propidium iodide-positive cells. Culturing OVCAR-3 cells in the presence of CA-MSC or BM-MSC CM before treatment with carboplatin resulted in, respectively, 43 \pm 4% and 45.5 \pm 6% of apoptosis versus 9 \pm 3% and 9 \pm 4% in untreated condition (Figures 1e and f and Supplementary Figure 1b). Notably, cells in early apoptosis (Annexin-V-positive only) represented 22 \pm 3% versus

37 before 4% in the control condition. Thus, carboplatin-induced early apoptosis in OVCAR-3 cells was significantly decreased in the presence of CA-MSC (Figures 1e and f). This observation was further validated by the decreased amount of PARP cleavage in response to carboplatin in the presence of CA-MSC or BM-MSC CM (Figure 1g and Supplementary Figure 1c). Notably, non-cancerous and non-mesenchymal cells such as HEK293

or fibroblast (CHN cells) CM did not affect carboplatin-induced apoptosis (Supplementary Figure 2). Using IGROV-1 cells, we also observed a decrease of carboplatin-mediated apoptosis in the cells cultured for 48h in the presence of CA-MSC CM (Supplementary Figure 3), whereas apoptosis was not modified in SKOV-3 cells (data not shown). These data suggested that CA-MSC- and BM-MSC-secreted factors could interfere with the apoptotic signalling pathway to promote ovarian cancer cell death resistance in response to carboplatin.

CA-MSC promote effector caspases activation defect.

Caspases are key actors in apoptosis signalling and a defect in their activation could be in part responsible for chemoresistance.³¹ To understand how CA-MSC-secreted factors could act on ovarian cancer cells to protect them from cell death, we investigated the molecular mechanisms of apoptosis and, in particular, the caspase activation cascade.

Carboplatin treatment decreased pro-caspase-8 and -9 expressions but enhanced their cleavage and catalytic activity in OVCAR-3 cells (Supplementary Figure 4). Incubation of the cells in the presence of CA-MSC CM did not affect carboplatin-induced caspase-8- and caspase-9-decreased

expression, cleavage and activation (Supplementary Figure 4). Concerning the effector caspases, carboplatin treatment decreased caspase-3 and -7 expressions but enhance their cleavage and catalytic activity in OVCAR-3 cells (Figures 2a and b). In contrast to the effect observed for the initiator caspases, CA-MSC CM prevented carboplatin-induced cleavage and catalytic activity of caspase-3 and -7 (Figures 2a and b). Interestingly, the expression of pro-caspase-3 and -7 was significantly decreased in the presence of CA-MSC CM without treatment (Figure 2a).

These results indicate that CA-MSC-secreted molecules perturb the executionary phase of apoptosis by inhibiting effector caspase activation upon carboplatin treatment.

CA-MSC secretions induce Akt and XIAP phosphorylation.

PI3K/Akt signal transduction has a critical role in the resistance to cisplatin through suppression of apoptosis in various types of human cancers including ovarian cancer.²⁴ We hypothesized that Akt activation and phosphorylation of its downstream targets could be involved in CA-MSC-induced apoptosis resistance in response to carboplatin. We tested the effect of CA-MSC CM on Akt phosphorylation in OVCAR-3 cells. Incubation of the cells during 24 h in the

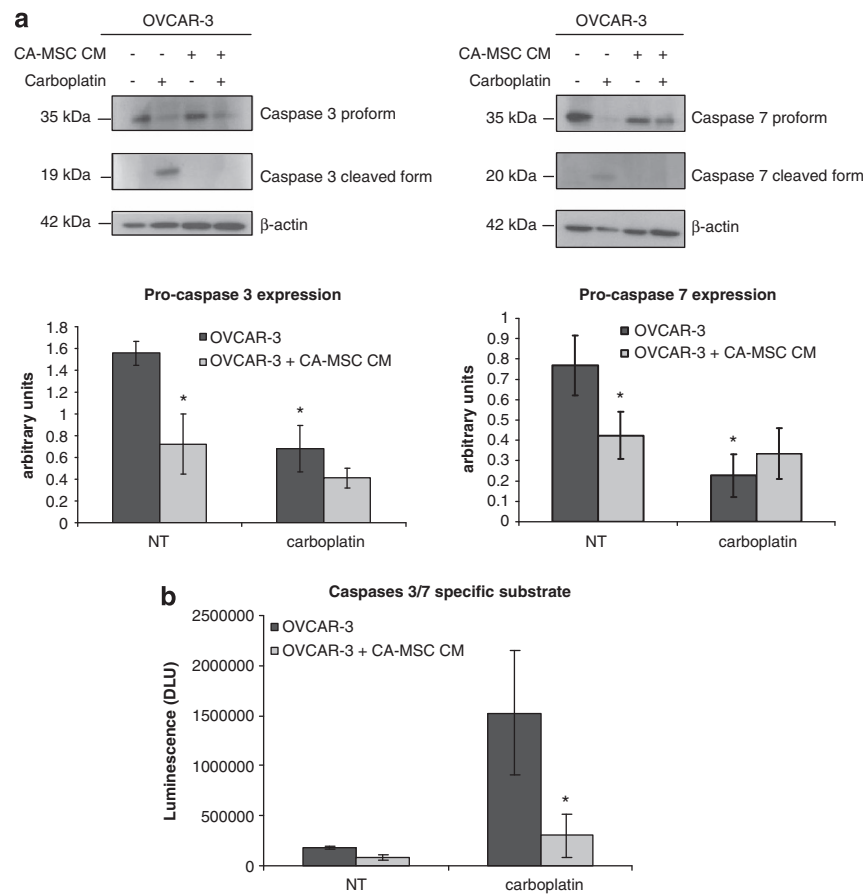


Figure 2 CA-MSC induce a downregulation of effector caspase-3 and -7 expression and inhibit their catalytic activation. OVCAR-3 cells cultured alone or in the presence of CA-MSC CM were treated with (+) or without (-) or (NT) 250 μ M carboplatin for 48 h. **(a)** Expression of caspases-3 and -7 was assayed by western blot. Pro-caspase-3 and -7 expression was normalized to β -actin expression (mean \pm S.E.M.; $n = 3$). * $P < 0.05$ versus not treated. **(b)** Catalytic activity of caspase-3 and -7 was assayed by measuring the luminescence emitted by caspase-3 or caspase-7 luminogenic-specific substrates, (RLU, relative lights units) (mean \pm S.E.M.; $n = 3$). * $P < 0.05$ versus not treated

presence of CA-MSC CM conditioned for 48 or 72 h promoted Akt phosphorylation on Ser473 (Figure 3a). We observed that the phosphorylation level increased with increasing conditioning time (Supplementary Figure 5). Notably, whereas PTEN level did not change, the level of total Akt decreased in the presence of CA-MSC CM (Figure 3a).

IAPs are a family of proteins that inhibit caspases and thereby regulate apoptosis.³² XIAP binds to and inhibits the effector caspases, that is, caspase-3, -7 and the initiator caspase-9. Dan *et al.*³³ showed that phosphorylation of XIAP on Ser87 by Akt stabilizes and protects XIAP from cisplatin-induced degradation.

We hypothesized that XIAP may be a target for phospho-Akt in our model. Thus, we evaluated the effect of CA-MSC CM on XIAP expression and phosphorylation in carboplatin-treated OVCAR-3 cells. Carboplatin treatment induced a decrease in XIAP expression but in OVCAR-3 cells that were exposed to CA-MSC CM before carboplatin treatment, we noticed a stabilization of XIAP (Figure 3b). XIAP stabilization could be associated with XIAP phosphorylation as we

observed an increase in the ratio of phosphorylated XIAP to total XIAP when the cells were cultured in the presence of CA-MSC CM (Figure 3b). We also evaluated the effect of CA-MSC CM on the expression of other IAPs family members (cIAP1, Livin and survivin). Whereas survivin expression did not change in the presence of CA-MSC CM, we observed a decrease in the expression of cIAP1 and Livin (Supplementary Figure 6).

These data show the phosphorylation of Akt as well as one of its downstream targets XIAP by CA-MSC-secreted factors. This suggests that CA-MSC secretion could activate the anti-apoptotic PI3K/Akt signalling. In order to confirm the link between the akt phosphorylation and the CA-MSC CM effect we evaluated carboplatin apoptosis induction in the presence or absence of CM and akt inhibitor. MK-2206 is a highly selective inhibitor of Akt1, Akt2 and Akt3. It inhibits auto-phosphorylation of both Akt T308 and S473. We prepared a CM as mentioned in the manuscript within 3 days. CM was added to the ovarian cancer cells in the presence or absence of 50 μ M of MK-2206 at day 0. At Day 1, carboplatin was added or not to the supernatants. We evaluated the amount of

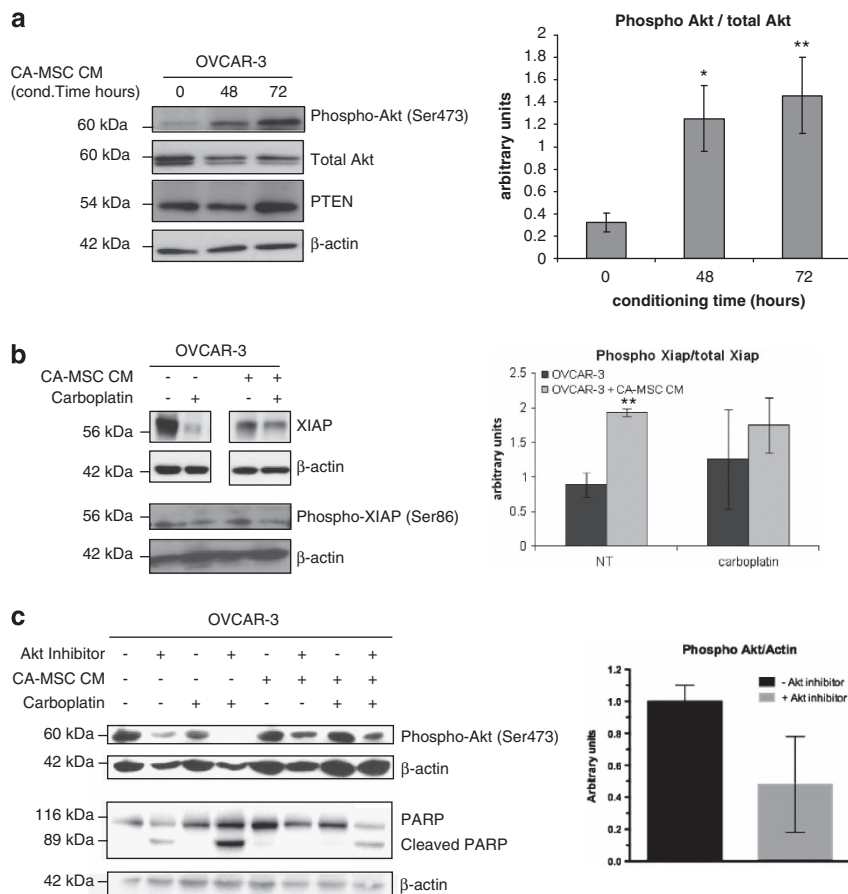


Figure 3 CA-MSC promote Akt phosphorylation and protect XIAP from cleavage upon carboplatin treatment. (a) OVCAR-3 cells were cultured with or without (0) CA-MSC CM conditioned for the indicated times. PTEN, Akt and phospho-Akt (Ser 473) expression was assayed by western blot. Phospho-Akt expression was normalized to total Akt expression (mean \pm S.E.M.; $n = 3$) * $P < 0.05$; ** $P < 0.01$ versus 0. (b) OVCAR-3 cells cultured alone (-) or in the presence of CA-MSC CM (+) were treated with (+ or carboplatin) or without (- or NT) 250 μ M carboplatin for 48 h. XIAP expression and phosphorylation were analysed by western blot. Phospho-XIAP expression was normalized to XIAP expression (mean \pm S.E.M.; $n = 3$). ** $P < 0.01$ versus OVCAR-3. (c) OVCAR-3 cells cultured alone or in the presence of CA-MSC CM were treated with (+) or without (-) with MK-2206 (selective inhibitor of Akt1, Akt2 and Akt3) for 48 h. Cells were treated also with (+) or without (-) 250 μ M carboplatin for the last 24 h. Phospho-Akt (Ser 473) expression as well as PARP cleavage was analysed by western blot

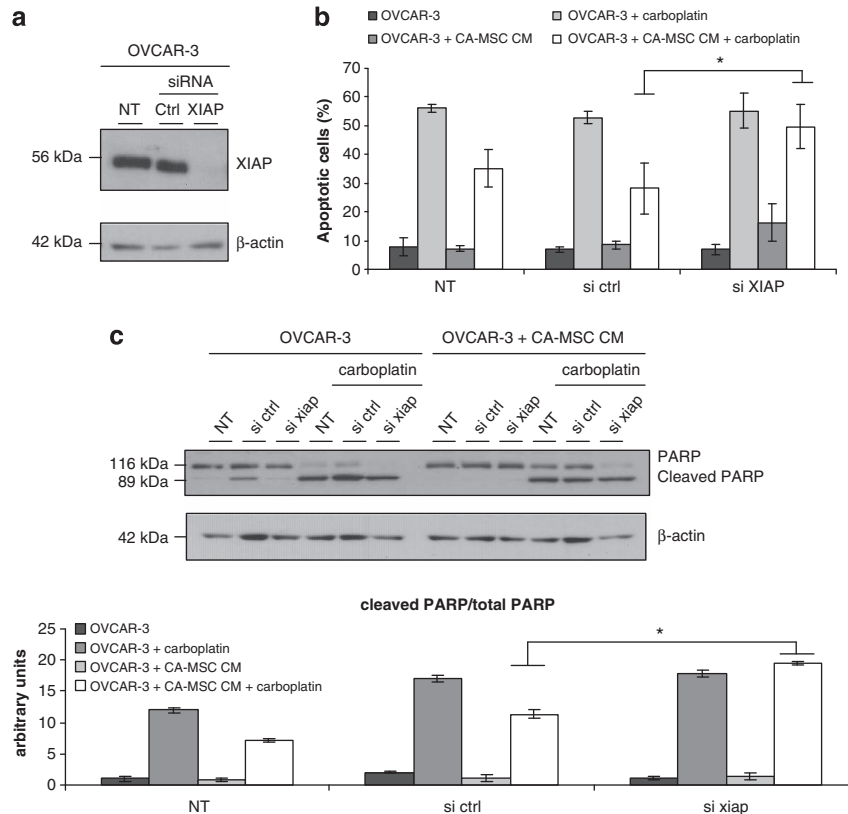


Figure 4 XIAP depletion restore carboplatin-induced apoptosis in ovarian cancer cells stimulated by CA-MSC. (a) OVCAR-3 cells were transfected or not (NT) with 20 nM of siRNA scramble (si ctrl) or XIAP-targeting siRNA (si XIAP) and 48 h after XIAP expression was analysed by western blot. (b and c) OVCAR-3 cells were transfected or not (NT) with 20 nM of siRNA scramble (si ctrl) or XIAP-targeting siRNA (si XIAP) in presence (+ CA-MSC CM) or absence of CA-MSC CM. Twelve hours after, cells were treated with or without carboplatin for 36 h. Apoptosis was measured by flow cytometry analysis after propidium iodide and Annexin-V-FITC labelling (b) and PARP cleavage (c). (b) Histogram representing the apoptotic cells population (Annexin-V-positive cells + Annexin-V/propidium iodide-positive cells) for each condition (mean \pm S.E.M.; $n = 3$). * $P < 0.05$. (c) PARP cleavage was analysed by western blot. Cleaved PARP level was normalized to total PARP level (mean \pm S.E.M.; $n = 3$). * $P < 0.05$

PARP cleavage in response to carboplatin in the presence of CA-MSC and the akt inhibitor (50% of inhibition of akt). As shown in Figure 3c, the akt inhibitor diminished the effect of the CA-MSC CM on apoptosis induction.

CA-MSC-secreted factors protect ovarian cancer cells from carboplatin-induced apoptosis through XIAP phosphorylation and stabilization. The modulation of XIAP expression regulating cisplatin sensibility in ovarian cancer cells^{24,26,27} and XIAP being a specific inhibitor of caspase-3 and -7,³⁴ we investigated the role of XIAP in the anti-apoptotic effect of CA-MSC CM.

We evaluated the effect of XIAP downregulation by siRNA on carboplatin-induced apoptosis in the presence or absence of CA-MSC CM. siRNA against XIAP totally inhibited XIAP expression evaluated by western blot (Figure 4a). In response to carboplatin, the inhibition of XIAP expression completely overcame cell death resistance induced by CA-MSC CM. Carboplatin-induced apoptosis and PARP cleavage were totally restored (Figures 4b and c).

These results indicate the involvement of XIAP in CA-MSC-induced carboplatin resistance in ovarian cancer cells.

Discussion

Chemoresistance is one of the most challenging problems in ovarian cancer as 70% of patients treated for ovarian cancer will relapse within 18 months after treatment with chemoresistant pathology. Despite the fact that the cancer cells are instable and could acquire mutations that will confer a chemoresistant phenotype, microenvironment could be involved in this phenomenon (for review see Castells *et al.*¹⁹). Among the microenvironment cells, we focused on MSC that are known to be recruited to tumour site and activated by cancer cells. It has been shown recently that they could either become CA-MSC or TAFs,^{11,35} both of them having some pro-tumoural properties. As there is no evidence on how MSC could be activated and become CA-MSC, we choose to study the role of both BM-MSC and CA-MSC in carboplatin resistance acquisition by ovarian tumour cells. Here we determined that both BM-MSC and CA-MSC demonstrated abilities to promote ovarian cancer cells chemoresistance. Our results indicate that this property does not seem to be induced by cancer cells contact or influence, and that naive MSC as well as activated MSC are able to confer chemoresistance to ovarian cancer cells.

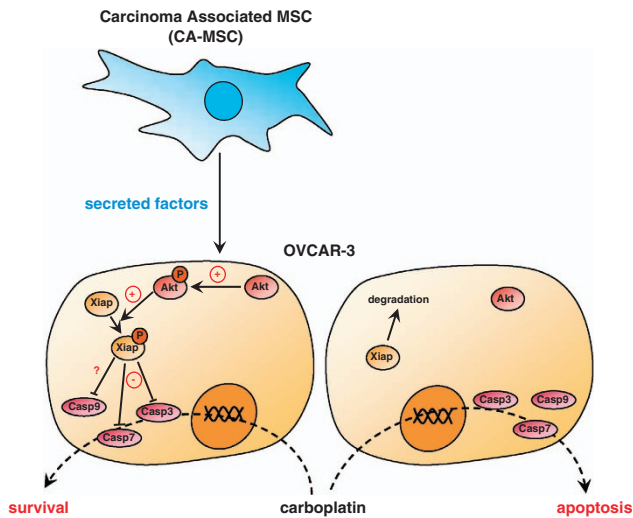


Figure 5 CA-MSC protect ovarian cancer cells from carboplatin-induced apoptosis through XIAP stabilization. Carboplatin triggers apoptosis in ovarian cancer cells via the activation of caspase-9, -3 and -7, and leads to the degradation of XIAP, an inhibitor of caspases. CA-MSC-secreted factor(s) activate Akt phosphorylation in ovarian cancer cells and permits XIAP phosphorylation promoting its stabilization. XIAP blocks apoptosis probably by inhibiting caspases-3 and -7 activation

As we already described that MSC *per se* are not able to increase tumour cells proliferation *in vitro*,¹⁵ we focused our study on their effect on apoptosis.

We demonstrated that both CA-MSC and BM-MSC increased ovarian cancer cells viability and prevented apoptosis in response to carboplatin treatment. The effects of MSC on chemoresistance acquisition have been already described by among others Rafii *et al.*¹⁷ who claimed that the anti-apoptotic effect of the MSCs was mediated through direct interactions between the MSC and the tumour cells. Here we showed that the effect of the MSC could be mediated through the secretions of factors in the culture medium. Indeed, all the experiments have been performed using CA-MSC- or BM-MSC CM. The identification of secreted factors responsible for the pro-tumoural effect of MSC is under investigation and will be discussed later. The factors involved in the phenomenon could be either a protein as a fatty acid, as Roodhart *et al.*⁶ recently published that fatty acids could be unregulated on MSC treated with carboplatin and that these fatty acids could be involved in the acquisition of chemoresistance by cancer cells.

Regarding to molecular mechanism of apoptosis, we determined that CA-MSC CM inhibited caspase activation, and, in particular, blocked effector caspase-3 and -7 proteolysis and subsequent activation, as well as their catalytic activity. We could reasonably relay the apoptosis inhibition effect of the MSC CM to the fact that caspase-3 activation is abrogated. As caspase-3 and -7 are activated by the initiator caspases, we checked the effect of CA-MSC CM on caspase-8 and -9 expression and activation, but we didn't notice any effect. However, in OVCAR-3 cells incubated in the presence of BM-MSC CM caspase-9 proteolysis and activation was affected, whereas CA-MSC CM did not affect this caspase. This suggests that CA-MSC do not have the same

properties as BM-MSC, concerning the released factors that could perturb the initiator caspases activation. Further, we checked for a regulator of apoptosis that could be involved in caspase-3 and -7 inhibition and subsequently apoptosis blockade.

The PI3K/Akt pathway activates survival and anti-apoptotic signalling. In ovarian tumours, activation of the PI3K/Akt pathway has been associated with aggressiveness of the tumour behaviour and increased survival (Dent *et al.*³⁶). In our model, we showed an activation of Akt, whereas PTEN expression was not affected, and CA-MSC CM promoted Akt phosphorylation on Ser473. We observed that the increase in the phosphorylated form of Akt increased as the conditioning time of CA-MSC medium increased (Figure 3a), suggesting the accumulation of secreted factors in CA-MSC CM acting on PI3K/Akt signalling. Preliminary results also indicated that CA-MSC stimulated Akt phosphorylation within a very short time (5 min; data not shown), indicating that CA-MSC may secrete growth factor that are able to bind to tyrosine kinase receptor. For instance, platelet-derived growth factor-AA, sphingosine 1-phosphate or prostaglandin E2 found in CA-MSC secretions (data not shown) could bind rapidly on their receptors at the surface of ovarian cancer cells and activate the PI3K/Akt signalling pathway.^{37–40} Moreover, a recent study from our group showed that CA-MSC promote IL-6 and interleukin-8 (IL-8) secretion in OVCAR-3 cells.⁷ IL-6 and IL-8 are known to activate survival signalling pathway and were recently involved in cisplatin resistance in ovarian cancer cells.^{41,42} Thus, secreted IL-6- and IL-8 could act in an autocrine manner on ovarian cancer cells surface receptors and also activate PI3K. Because OVCAR-3 cells do not secrete these cytokines, another factor in CA-MSC CM may activate IL-6 and IL-8 synthesis and secretion in these cells, thereby delaying the activation of PI3K/Akt signalling. Thus, we can speculate that growth factors secreted by CA-MSC are responsible for the early activation of Akt, and later CA-MSC-induced IL-6 and IL-8 secretion by ovarian cancer cells sustain the activation of PI3K. Among the downstream target of Akt, we observed the phosphorylation and stabilization of XIAP after carboplatin treatment in OVCAR-3 cells priorly incubated in the presence of CA-MSC CM. XIAP is able to bind and inhibit the initiator caspase-9 and the effector caspase-3 and -7. Thus, the inhibition of effector caspases in the presence of CA-MSC CM could be attributed to XIAP through its phosphorylation by Akt. However, caspase-9 expression and activation were not affected by CA-MSC CM (Figure 5). This could be explained by the fact that binding domains of XIAP are different for initiator and effector caspases. Indeed, the BIR2 domain of XIAP inhibits caspase-3 and -7, whereas BIR3 binds to and inhibits caspase-9.⁴³

We cannot exclude the potential phosphorylation of other molecules by Akt in our model. So far, we did not observe any difference in the phosphorylation of caspase-9, Bcl-xL and Bcl-2 in the presence of CA-MSC CM (Supplementary Figure 7), suggesting that the mitochondrial targets of Akt are not involved in apoptosis resistance in our model. However, to confirm the involvement of Akt, we have investigated the effect of the inhibition of Akt phosphorylation on CA-MSC-induced carboplatin resistance in OVCAR-3

cells, and showed that the akt inhibition abrogated the effect of the CA-MSC CM on apoptosis induction.

Interestingly, we found a downregulating effect of CA-MSC CM on the expression of apoptotic (pro-caspase-3 and -7) and anti-apoptotic proteins (total Akt, cIAP and Livin) in OVCAR-3 cells (Figures 2a and 3b and Supplementary Figure 6). For all of them, the observed decrease in the protein level was detected after the incubation of OVCAR-3 cells in the presence of CA-MSC CM during 24 h. These observations suggested a genetic regulation of the expression of these proteins by CA-MSC-secreted factors.

As XIAP is a current target for the treatment of chemo-resistant cancer such as colon cancer,⁴³ we verified whether XIAP was involved in the apoptosis resistance observed in ovarian cancer cells exposed to CA-MSC CM. XIAP depletion by siRNA in OVCAR-3 cells exposed to CA-MSC CM completely restored the sensitivity of the cells to carboplatin. These results indicate a key role of XIAP in CA-MSC-mediated apoptosis resistance in ovarian cancer cells. Some preliminary data provided by Genre *et al.* (manuscript in preparation) showed that the XIAP-inhibiting SMAC mimetics DEBIO1143, developed by DEBIOpharm^{44,45}, is able to counteract the action of the CM by reverting the chemoresistance acquisition induced by the CM (data not shown).

To conclude, our study underlines the role of XIAP in apoptosis protection induced by CA-MSC secretions in ovarian cancer cells. The potential use of XIAP inhibitors could overcome cancer cells resistance to chemotherapy mediated by the microenvironment. Molecules secreted by CA-MSC deserve a full characterization and will bring a better understanding of both microenvironment cancer cells cross talk and mechanism leading to chemoresistance acquisition in ovarian cancer model.

Materials and Methods

Cell culture. HOSpicells (CA-MSC)^{15,46,47} and CHN cells (human corneal fibroblasts⁴⁸) were obtained from M. Mirshahi (INSERM UMR 736). HOAC lines OVCAR-3 and SKOV-3 cells (ATCC Numbers HTB-161 and HTB-77) were obtained from the American Type Culture Collection (Manassas, VA, USA). HOAC line IGROV-1 was a gift from the Institut Gustave Roussy, Villejuif, France.^{49,50} HEK293 cells were from qBiogen (Illkirch, France). BM-MSC were obtained from bone marrow of healthy donors and were obtained from Etablissement Français du Sang. MSC were immortalized by SV40 large T antigen. The CA-MSC that we used here present a phenotype related to the MSC because they have been isolated from their high affinity to the tumoural cells and because they were negative for CD45, CD34 and HLA-DR surface markers, and positive ($\geq 97\%$) for CD9, CD10, CD29, CD146, CD166, HLA1 and CD13. They express Tenascin-C.¹⁹ Moreover, when injected in tumour-bearing animal, they migrate through the tumour cells and to the inflammation sites. They have anti-immunogenic properties⁵¹ and pro-tumoural activities.¹⁵

All cells were cultured in RPMI medium supplemented with 10% foetal calf serum, penicillin/streptomycin (100 IU/ml/100 μ g/ml) and 2 mM L-Glutamine (Cambrex Biosciences, Milan, Italy). Cell lines were routinely checked for mycoplasma.

Conditioned media. CA-MSC (0.5×10^6) or BM-MSC were seeded in 100-mm diameter Petri dishes (Falcon, Becton Dickinson, Le Pont-de-Claix, France) in RPMI with 10% of foetal calf serum for 72 h. The CM were then collected, 0.22 μ m filtered, and used to seed HOAC for experiments.

IC50 assay. HOAC were seeded in 96-well plates (5000 cells per well) in the presence or absence of CA-MSC- or MSC CM and treated 24 h after with the indicated concentrations of carboplatin. After 48 h, cell viability was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, L'Isle d'Abeau Chesnes, France) assay. Untreated cells were used as 100% of viability.

Annexin-V-FITC and propidium iodide staining. HOAC were seeded in six-well plate (1×10^5 cells per well) in the presence or absence of CA-MSC or MSC CM. After 24 h, cells were treated with 250 μ M carboplatin for 48 h and then harvested for co-staining with FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA) following instructions of the manufacturer. Analyses were performed on a FACS Calibur flow cytometer (BD Biosciences, Le Pont-de-Claix, France) and CellQuest software (BD Biosciences).

Western-blotting analysis. Fifty micrograms of proteins were separated by SDS-PAGE on a 10% polyacrylamide gel. Proteins were transferred on a methanol-activated PVDF membrane. Membranes were saturated for 1 h in TBS (50 mM Tris and 150 mM NaCl)/0.1% Tween 20/5% milk or 5% bovin serum albumin for phosphorylated proteins analysis and incubated overnight at 4 °C with a rabbit polyclonal primary antibody directed against caspase-8 (1:1000, Santa Cruz, Nanterre, France, clone H-134), phospho-Xiap (Ser87) (1:1000, Abcam, Paris, France), phospho-caspase-9 (Ser96) (1:100, Abgent clone RB6898); Bcl-xl (clone Ab-62), phospho-Bcl-xl (Ser62), Bcl-2 (clone Ab-56), phospho-Bcl-2 (Thr56) (1:1000, Genscript, Paris, France); PARP, caspase-9, caspase-3, caspase-7, phospho-Akt (Ser473) (clone D9E), Akt (clone C67E7), XIAP (clone 3B6), XIAP pSer87 ref 0599 (Assay Biotech (Interchim), Montluçon, France), CIAP1, livin (clone D61D1), survivin (clone 6E4) (1:1000, Cell Signalling Technology (Ozyme), Saint Quentin, France) or a monoclonal primary antibody directed against actin (1:1000, Cell Signalling Technology). Membranes were washed three times with TBS/0.1% Tween 20 (TT) and then incubated for 1.5 h with a secondary antibody (1:4000 anti-rabbit or anti-mouse, Cell Signalling Technology) coupled with horseradish peroxidase (HRP). Membranes were washed three times with TT. Immunocomplexes were revealed by ECL (Pierce (Perbio), Brebières, France).

Caspase activation assay. OVCAR-3 cells were seeded in 96-well plate (5000 cells per well) in the presence or absence of CA-MSC or MSC CM. After 24 h, cells were treated with 250 μ M carboplatin for 48 h and caspase activation was monitored using luminescence-based caspase-Glo kit (Promega, Charbonnières les bains, France) following instructions of the manufacturer. Luminescence from cleaved caspase substrate was quantified using a luminometer (Berthold, Thoiry, France).

Akt inhibition. MK-2206 (Selleckchem) is a highly selective inhibitor of Akt1, Akt2 and Akt3. It inhibits phosphorylation of both Akt T308 and S473. Cells were treated with 50 μ M of MK-2206 in the presence or absence of CM prepared as mentioned previously. After 24 h, cells were treated with 250 μ M carboplatin for 24 h. Cell proteins were extracted at day 2.

Transfection. Cells incubated in the presence or absence of CA-MSC CM were transfected with lipofectamine RNAiMAX transfection reagent (Invitrogen, Cergy-Pontoise, France) and the following siRNA against XIAP (Qiagen, Courtabouef, France): 5'-GUGCUUUUCACUGUGGAGGATT-3', according to the manufacturer's protocols. siRNA allStars Negative control siRNA (Qiagen) were used as negative controls.

Twelve hours after transfection, cells were treated with 250 μ M carboplatin for 36 h and further analysed by flow cytometry or western blot.

Statistical analysis. Results were expressed as the mean \pm S.E.M. Student's two-sided *t*-test was used to compare values of test and control samples. **P* < 0.05 and ***P* < 0.01 indicated a significant difference.

Conflict of Interest

The authors declare no conflict of interest.

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