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Gemcitabine-releasing mesenchymal stromal cells inhibit *in vitro* proliferation of human pancreatic carcinoma cells

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Abstract

Background aims. Pancreatic cancer (pCa) is a tumor characterized by a fibrotic state and associated with a poor prognosis. The observation that mesenchymal stromal cells (MSCs) migrate toward inflammatory micro-environments and engraft into tumor stroma after systemic administration suggested new therapeutic approaches with the use of engineered MSCs to deliver and produce anti-cancer molecules directly within the tumor. Previously, we demonstrated that without any genetic modifications, MSCs are able to deliver anti-cancer drugs. MSCs loaded with paclitaxel by exposure to high concentrations release the drug both *in vitro* and *in vivo*, inhibiting tumor proliferation. On the basis of these observations, we evaluated the ability of MSCs (from bone marrow and pancreas) to uptake and release gemcitabine (GCB), a drug widely used in pCa treatment. *Methods.* MSCs were primed by 24-h exposure to 2000 ng/mL of GCB. The anti-tumor potential of primed MSCs was then investigated by *in vitro* anti-proliferation assays with the use of CFPAC-1, a pancreatic tumor cell line sensitive to GCB. The uptake/release ability was confirmed by means of high-performance liquid chromatography analysis. A cell-cycle study and secretome evaluation were also conducted to better understand the characteristics of primed MSCs. *Results.* GCB-releasing MSCs inhibit the growth of a human pCa cell line *in vitro*. *Conclusions.* The use of MSCs as a "trojan horse" can open the way to a new pCa therapeutic approach; GCB-loaded MSCs that integrate into the tumor mass could deliver much higher concentrations of the drug *in situ* than can be achieved by intravenous injection.

Key Words: *drug delivery, gemcitabine, MSCs, pancreatic adenocarcinoma*

Introduction

Recently, the approach to studying the biology of cancer has been changed by the new concept of cancer as an "anomalous organ" rather than simply a "tumor mass" [1]. Indeed, this is a more complete and integrated perspective because it considers the interaction between cancer cells and the different tissue components within the tumor mass (vascular system, stromal and inflammatory cells, extracellular matrix [ECM]) that probably form a critical micro-environment for tumor development [2]. These cell populations play different important roles, depending on the tumor type, and the relationship among them may influence cancer growth [3]. Fibroblasts, inflammatory cells (eg, lymphocytes and macrophages), endothelial cells, pericytes, smooth muscles and other cells may have synergic effects on tumor progression. In this context, for example, large differences may exist between the normal and the tumor stroma and could play a decisive role in tumorigenesis and development [4,5].

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Our study is focused on pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer and the fourth leading cause of cancer death in the United States [6]. PDAC is very aggressive and has a poor prognosis. Furthermore, its resistance to chemotherapy and radiotherapy limits the efficacy of these therapeutic approaches. Currently, PDAC is a deadly disease; the overall 5-year survival rate is approximately 5% because of the difficulty of early diagnosis, the highly aggressive nature of the disease and the lack of effective therapies [7]. After surgical curative resection (<20%), only approximately 25% survive to 5 years because of the high rate of local and metastatic recurrence [7,8]. Gemcitabine (GCB) and 5-fluorouracil are the current first-line chemotherapies for locally advanced and metastatic PDACs; however, these treatments achieve a clinical response of only 10% [9−11]; hence, novel therapies are urgently needed.

It is well known that PDAC is characterized by the proliferation of stromal fibroblasts and deposition of ECM, giving rise to a fibrotic state known as desmoplastic or reactive stroma. PDAC is associated with poor prognosis [1] because of the propensity of early metastasis and high resistance to both chemotherapy and radio-therapy [12]. Furthermore, desmoplastic stroma contains small endothelium-lined vessels and inflammatory cells that are not residual atrophic components of parenchyma of the invaded organ [13]. Several components might contribute to the fibroblast population: stellate cells, peri-vascular fibroblasts and bone marrow (BM)-derived cells; all of them are activated by a tissue injury and accumulate in the pancreas during carcinogenesis [14,15].

In the recent years, the ability of MSCs to migrate toward inflammatory micro-environments and engraft into tumor sites after systemic administration [16] have led to the development of new therapeutic approaches that are based on the cell-based delivery of anti-cancer agents by MSCs, either gene-modified or not gene-modified. Some authors [17,18] engineered MSCs from different sources, such as the adipose tissue and pancreas, respectively, to arm them with tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), an anti-cancer death molecule able to induce apoptosis in several tumor types. Recently, we have demonstrated that MSCs are able to deliver anti-cancer agents without any genetic modifications [19−21]. Indeed, when exposed to high doses of the chemotherapeutic drug paclitaxel, MSCs accumulated drug intracellularly and then released it, reducing tumor proliferation both *in vitro* and *in vivo*. To expand this line of study, in the present study we evaluated the ability of BM- or pancreas-derived MSCs to uptake GCB, the first-line chemotherapeutic drug for pCa treatment [22].

We found that once loaded with GCB, MSCs were able to release it into the culture medium, becoming GCB-releasing-MSCs (MSCsGCB) that inhibited the *in vitro* growth of a human adenocarcinoma cell line.

Methods

Bone marrow MSCs

BM-MSCs were prepared from the mononuclear cell fraction of human BM, which was purchased frozen in liquid nitrogen from Lonza and stored at $-120°C$ until use. After thawing, cells were suspended in Dulbecco's modified Eagle's medium with low glucose, 10% fetal bovine serum (FBS) (EuroClone), 2% L-glutamine and 10 ng/mL basic fibroblast growth factor (bFGF, ReliaTech), plated in 25-cm^2 flasks (Corning) at 2×10^6 cells/mL and incubated at $37°C$, 5% CO_2. After 48 h, floating cells were discarded; medium was replaced and was then changed weekly until cells reached 80% confluency. The BM-MSC monolayer was then trypsinized; cells were seeded at the density of 5000 cells/cm^2 and used until passage 3.

Pancreas-derived MSCs

Primary human pancreatic tissues were obtained from the digest remaining after the isolation of islet cells from human pancreas, as previously described [23]. The dense fraction recovered in the pellet and normally discarded was processed for MSC isolation. After two washes in phosphate-buffered saline (PBS), the equivalent of a 1-mL packed pellet was re-suspended in α-minimum essential medium (MEM) with 10% FBS, plated in one T75 tissue culture−treated flask (Costar) and grown at $37°C$ in a humidified incubator at 5% CO_2. After 24 h, the non-adherent material was removed and fresh medium was added to the cells. Medium was changed every 3 days. Cells were expanded in α-MEM with 10% FBS, 1% L-glutamine and 10 ng/mL bFGF, and, when 80% confluency was reached, were trypsinized and seeded at the density of 8000 cells/cm^2. In all the experiments, MSCs were used until passage 10.

GCB priming of MSCs

GCB hydrochloride was purchased from Accord Healthcare Limited. After reconstitution in 0.9% sodium chloride injection, the stock solution (38 mg/mL) was stored at $-20°C$. On the day of experiments, GCB was thawed and diluted in culture medium to the required concentration.

Sub-confluent MSC cultures ($3−4 \times 10^5$ cells) were exposed to 2000 ng/mL of GCB. Twenty-four hours later, the cells were washed with PBS, trypsinized, washed twice, and, after the evaluation of their

number and viability, seeded in a new flask at the concentration of 10^5 cells/mL. Forty-eight hours later, the cell-conditioned media (CM) from primed MSCs (MSCsGCB-CM) were collected, centrifuged at 2500*g* for 15 min to discard cell debris, aliquoted and stored at $-70°C$.

The remaining cells were trypsinized and then lysed (MSCsGCB-LYS, see Supplementary Information). Both CM and LYS were tested for their *in vitro* anti-proliferative activity against CFPAC-1, a human PDAC cell line sensitive to GCB [24,25]. CM and LYS from untreated MSCs were used as negative controls. CM from primed as well as control MSCs were analyzed for their cytokine/chemokine content by use of multiplex bead−based assays on xMAP technology (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Plex Human Group II Cytokine 21-Plex Panel; Biorad Laboratories).

Cell cycle and population doubling time (PDT) were evaluated to compare untreated cells and 24-h GCB-primed cells. Briefly, cells were suspended in PBS and fixed with 96% (vol/vol) ethanol for 1 h at $4°C$. After PBS wash, cells were suspended in propidium iodide 50 µg/mL in PBS, incubated overnight at $4°C$ and analyzed by means of flow cytometry (FacsVantageSE, Becton-Dickinson). For the evaluation of PDT, see Supplementary Information.

In vitro anti-proliferative assay on CFPAC-1 of GCB, CM and LYS from MSCsGCB

The effects of GCB, MSCsGCB-CM and MSCsGCB-LYS were studied on CFPAC-1 in 96-multi-well plates (Greiner Bio-One) with the use of the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium (MTT) assay as previously described [19]. The inhibitory concentrations (IC_{50} and IC_{90}) were determined according to the Reed and Muench formula [26]. The anti-tumoral activities of MSCsGCB-CM and MSCsGCB-LYS were compared with pure GCB and expressed as gemcitabine equivalent concentration (G.E.C.) according to the following algorithm: G.E.C. (ng/mL) = $100/V_{50}*IC_{50}$GCB. V_{50} is the volume (µL/well) of MSCsGCB-CM or -LYS able to inhibit the proliferation of CFPAC-1 by 50%; IC_{50}GCB is the concentration (ng/mL) of pure GCB producing 50% of inhibition. G.E.C., referred to a single primed MSC, was calculated as the ratio between the total amount of equivalent GCB and the number of cells seeded: G.E.C. (pg/cell) = G.E.C. (ng/mL) × CM or LYS volume (mL) × 1000/number of cell seeded.

Direct in vitro inhibition of CFPAC-1 by MSCsGCB

To verify the ability of MSCs to inhibit the *in vitro* CFPAC-1 proliferation, a co-culture assay was performed by mixing 1000 tumor cells (TCs) with eight different amounts of MSCs (4000−2000−1000−500−250−125−63) to achieve a final proportion MSCs/CFPAC-1 of 4:1−2:1−1:1−1:2−1:4−1:8−1:16. This assay, conducted in duplicate in 96-multi-well plates, was performed on three experimental conditions: (i) mixing CFPAC-1 and untreated MSCs; (ii) mixing CFPAC-1 with MSCs primed with GCB (MSCsGCB); (iii) CFPAC-1 (1000 cells/well only) and MSCs alone (both normal and primed) at the eight different amounts. The cultures were incubated for 7 days in 95% air + 5% CO_2 at $37°C$; cell growth was then evaluated by use of the MTT assay. The results were expressed as a percentage of the proliferation observed in TCs culture that did not receive MSCs (considered as 100%). The arbitrary value of R_{50} was calculated as the ratio of CFPAC-1/MSCs able to inhibit TC proliferation by 50%.

Evaluation of anti-angiogenic properties of MSCsGCB

To assess the anti-angiogenic potential of MSCs and MSCsGCB, initially we tested the effect of GCB and CM (collected after 48 h of culture from control and GCB-primed MSCs) on the proliferation of human umbilical vein endothelial cells (HUVECs) (Lonza). HUVECs were routinely maintained in an endothelial cell growth medium bullet kit (EGM) plus 10% fetal calf serum (Lonza). Endothelial cell proliferation assay was performed as follows. Briefly, HUVECs at passage 3 were harvested by use of trypsin, then re-suspended in EGM + 0.2% bovine serum albumin and counted. To evaluate the growth response to CM from control and GCB-primed MSCs, 0.5 mL of HUVECs (10^4 cells) were seeded into each well of a 24-multi-well plate coated with collagen type I; after cell adhesion, medium was aspirated and replaced with EGM complete medium supplemented or nonsupplemented with different dilution (ranging from 1:2 to 1:8) of MSCs-CM and MSCsGCB-CM. Negative and positive controls were HUVECs grown in the basal medium (EBM), respectively, without and with addiction of supplements. Furthermore, the activity of GCB (from 10 to 1000 ng/mL) on HUVEC proliferation was evaluated both in the basal and supplemented medium. After 72 h, the wells were washed, fixed and stained. The cells were counted with a calibrated ocular eyepiece in 10 different fields at 40× magnification.

Statistical analysis

Data tested for normality have been analyzed by means of different statistical assays. Differences between two means were evaluated according to the
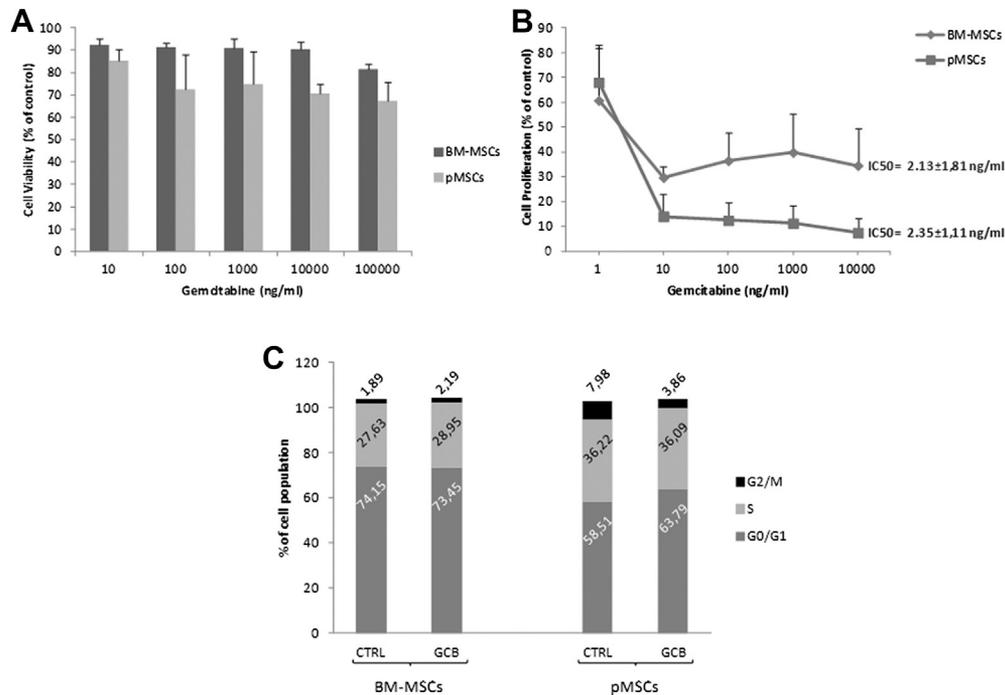
Figure 1. GCB sensitivity of BM-MSCs and pMSCs. The cytotoxic and anti-proliferative effects of GCB were evaluated by means of MTT assay by culturing MSCs for 24 h (A) or 7 days (B) in the presence of increasing logarithmic concentrations of drug. The effects are expressed, respectively, as cell viability and cell proliferation (percentages of the optical density measured in cultures that did not receive GCB, considered as 100%). The reported values (mean ± standard deviation) refer to one experiment performed in quadruplicate (cytotoxicity) and to at least two experiments performed in triplicate (anti-proliferation). (C) Cell-cycle phase distribution analysis of MSCs exposed to 2000 ng/mL of GCB for 24 h (CTRL indicates control cells, non-primed MSCs). Each value is the mean of two experiments.

Student's t-test; an analysis of variance test was applied for comparing three or more groups. Two-tailed P values >0.05 were not considered significant.

Results

Ex vivo expansion of MSCs and their characterization

The flow cytometry analysis and the differentiation assays (osteogenesis, adipogenesis and chondrogenesis) confirmed the mesenchymal/stromal phenotype of BM-MSCs and pancreas-derived MSCs (pMSCs). Reverse-transcriptase−polymerase chain reaction analysis revealed the expression of the sodium-dependent concentrative nucleoside transporter 1 (hCNT-1, Supplementary Figure S1) both in BM-MSCs and pMSCs.

Cytotoxic and anti-proliferative activity of GCB to MSCs

In the 24-h cytotoxicity assay (Figure 1A), both BM-MSCs and pMSCs showed a very high resistance to GCB; even at the highest GCB concentration tested (100,000 ng/mL), we observed a 20% to 30%

reduction of cell viability. On the basis of these data and the previous procedure developed with paclitaxel [19], we selected to prime MSCs with 2000 ng/mL for 24 h. These conditions were considered suitable for GCB and both types of MSCs because (as confirmed by the cell-cycle analysis and the PDT calculation) cell proliferation was almost completely blocked but cell viability was not substantially affected. Indeed, if primed cells were sub-cultured for 144 h after MSCsGCB-CM collection, the viability values were 75.55% ± 8.11% for BM-MSCsGCB and 91.20% ± 4.53% for pMSCsGCB (Supplementary Figure S2).

The cell-cycle analysis showed that high GCB concentrations were able to produce a total arrest of cell-cycle progression in all phases and not, as expected, a cell-cycle arrest in S phase only. Indeed, after incubating both BM-MSCs and pMSCs for 24 h in 2000 ng/mL GCB, the percentages of cells in G1, S and G2/M were not significantly modified in comparison to control samples.

The sensitivity of MSCs to the anti-proliferative effect of GCB was assessed in a 7-day anti-proliferation assay (Figure 1B). BM-MSCs and pMSCs showed similar sensitivity, with IC_{50} values,

A

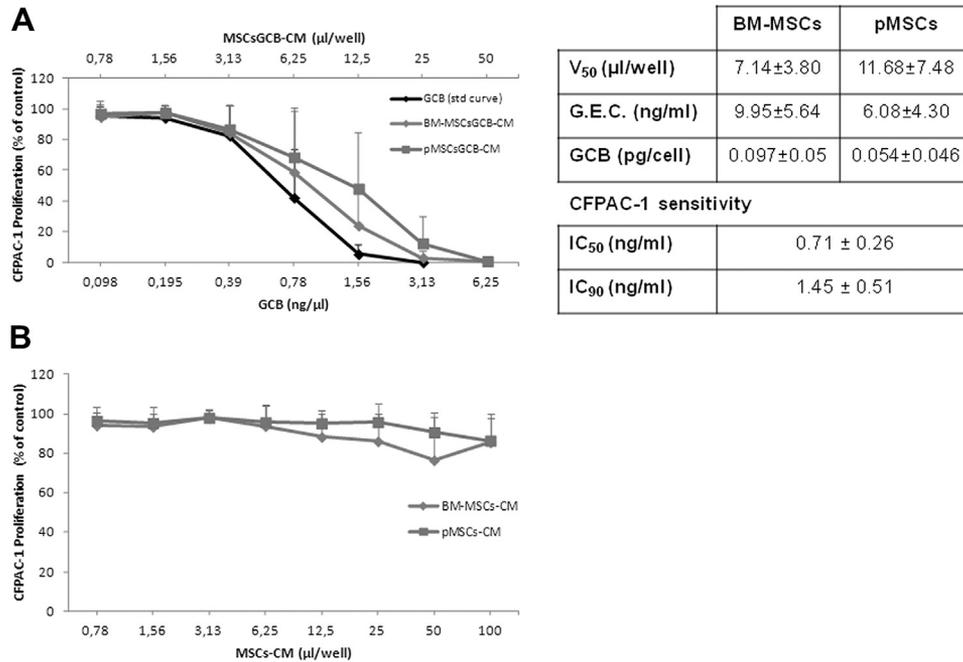|	BM-MSCs	pMSCs
V_{50} (µl/well)	7.14±3.80	11.68±7.48
G.E.C. (ng/ml)	9.95±5.64	6.08±4.30
GCB (pg/cell)	0.097±0.05	0.054±0.046
CFPAC-1 sensitivity		
IC_{50} (ng/ml)	0.71 ± 0.26	
IC_{90} (ng/ml)	1.45 ± 0.51	

B

Figure 2. Anti-proliferative activity of MSCsGCB-CM on CFPAC-1. (A) Inhibitory activity of BM-MSCsGCB-CM and pMSCsGCB-CM evaluated in CFPAC-1 standard assay in comparison to the standard drug. Tables on the right show V_{50} values, expressing the CM volume (µL/well) able to produce a 50% inhibition of CFPAC-1 proliferation: this parameter, together with the IC_{50} value (ng/mL) assessed with the pure drug, allows estimation of the GCB concentration in MSCsGCB-CM (G.E.C) and the amount of the drug released by a single primed MSC, expressed as picograms (pg)/cell (see text for detailed equation). Results are expressed as percentages of the optical density measured in CFPAC-1 grown in culture medium (for standard GCB curve) or CFPAC-1 grown in MSCs-CM (conditioned media collected from non-primed MSCs, for MSCsGCB-CM). As shown by (B), CM collected from non-primed MSCs were ineffective on CFPAC-1 proliferation. Values are expressed as mean ± standard deviation of at least three independent experiments.

respectively, of 2.13 ± 1.81 and 2.35 ± 1.11 ng/mL. Surprisingly, 10 ng/mL of GCB reduced cell proliferation by 70% to 80%, but higher concentrations did not increase the anti-proliferative effect.

Evaluation of drug release from MSCsGCB on pancreatic carcinoma cells

The activity of GCB released by MSCs was tested on CFPAC-1, a human PDAC cell line very sensitive to GCB (IC_{50} = 0.71 ± 0.26 ng/mL) (table in Figure 2). MSCsGCB-CM produced a strong, concentration-dependent anti-proliferative effect on CFPAC-1, equivalent to that obtained with pure GCB tested from 0.098 to 3.13 ng/mL (Figure 2A). As demonstrated by the V_{50} values reported in the box of Figure 2 (7.14 ± 3.80 for BM-MSCs versus 11.68 ± 7.48 for pMSCs), no significant difference in efficacy was observed between CM from BM-MSCsGCB and pMSCsGCB. The CM from control MSCs (non-primed with GCB) were ineffective at inhibiting CFPAC-1 proliferation (Figure 2B).

By comparing the activity of 2-fold serial dilutions of MSCsGCB-CM with the inhibitory activity of pure GCB on CFPAC-1, we calculated an equivalent GCB concentration (G.E.C.), and, with

the use of the value with the number of primed cells, it was possible to estimate the amount of drug released by a single primed MSC (expressed as pg/cell). The values reported in the box of Figure 2 confirm that MSCs derived from BM and pancreas have the same ability to uptake and release amounts of GCB that are effective against TCs.

In vitro direct anti-proliferative activity of MSCsGCB on CFPAC-1

The capacity of drug-loaded cells (MSCsGCB) to inhibit TC proliferation by direct action was confirmed by use of a co-culture assay (MSCsGCB and CFPAC-1 mixed at different ratios). As expected, the MSCsGCB inhibited the proliferation of CFPAC-1 according to their proportional presence (Figure 3A and Supplementary Figure S5a). An R_{50} value was calculated to represent the ratio (CFPAC-1/MSCsGCB) able to reduce pancreatic carcinoma proliferation by 50%. For BM-MSCs, R_{50} was 3.82 ± 2.31 and for pMSCs, 3.1 ± 0.42, whereas MSCs without loaded drug inhibited CFPAC-1 proliferation, with 50% inhibition occurring only at very high ratios of BM-MSCs (R_{50} = 0.25 = 1/4). The direct inhibitory potential of MSCsGCB in co-culture is
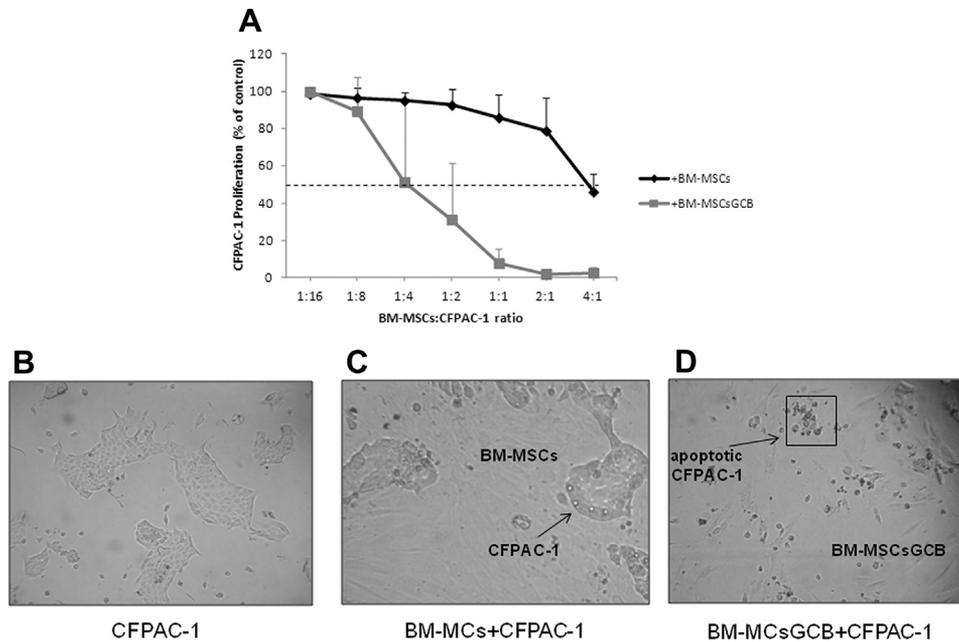
Figure 3. *In vitro* direct anti-proliferative activity of BM-MSCsGCB on CFPAC-1. The ability of BM-MSCsGCB to directly inhibit the proliferation of CFPAC-1 was evaluated by use of a co-culture assay in a 96-well plate: 1000 tumor cells were mixed with different amounts of BM-MSCs, both control and GCB-primed, to have seven different ratios BM-MSCs:CFPAC-1 (A). After a 7-day incubation, the proliferation of CFPAC-1 was evaluated by use of MTT assay and expressed as percentage of the optical density (O.D.) measured for CFPAC-1 cultured without BM-MSCs (◆) or O.D. measured for CFPAC-1 cultured with non-primed BM-MSCs (■). Values are expressed as mean ± standard deviation of at least two independent experiments performed in duplicate. (B, C, D) CFPAC-1, BM-MSCs+CFPAC-1 (1:1) and BM-MSCsGCB+CFPAC-1 (1:1) cultures, respectively.

shown in Figure 3 and Supplementary Figure S5, in which the arrows indicate groups of CFPAC-1 cells damaged by the GCB released by the co-cultured loaded MSCs.

Effect of MSCsGCB-CM on endothelial cell proliferation

As reported in Figure 4A, GCB was able to inhibit HUVEC proliferation at three concentrations of 1000, 100 and 10 ng/mL. This inhibitory effect was particularly evident when HUVECs were cultured in EGM medium. In the absence of supplements (EBM + 10% FBS, the control medium), endothelial cells had a very low growth rate and the inhibitory activity of GCB was not significant (compared with cells grown without drug). CM derived from GCB-primed BM-MSCs (Figure 4B) and pMSCs (Figure 4C) caused significant inhibition of HUVEC proliferation in a concentration-dependent manner, whereas CM from control MSCs did not inhibit HUVEC proliferation.

Discussion

Our data demonstrated for the first time that MSCs derived both from BM and pancreas can be loaded

in vitro with GCB. Previously, we demonstrated the ability of MSCs from different sources [19–21] to accumulate and release PTX, a drug having a mechanism of action compatible with many physiological functions of the MSCs. Indeed, PTX binds to a specific target in the MSC cytoskeleton, producing a block of cell proliferation without affecting viability or drug delivery. This new study on GCB uptake/ release by MSCs is very important because of the wide use of this drug in treatment regimens for pancreatic carcinoma [22].

A GCB loading concentration of 100,000 ng/mL was only modestly cytotoxic (20% to 30%) to MSCs derived from either BM or pancreas. This agrees with the report by Schmidmaier [27] that GCB did not affect BM-MSC viability up to 330 ng/mL. As expected, lower GCB concentrations (2.13 ± 1.81 ng/ mL for BM-MSCs and 2.35 ± 1.11 ng/mL for pMSCs) blocked cell proliferation by 50%. GCB concentrations higher than 10 ng/mL do not cause any further changes in MSC proliferation, probably because of the block in cell division (as confirmed by cell-cycle analysis and PDT calculation) despite the high number of viable cells (MTT assay). The results obtained by means of cell-cycle analysis agree with the observation that low concentrations of GCB induce cell-cycle arrest in the S phase, whereas high
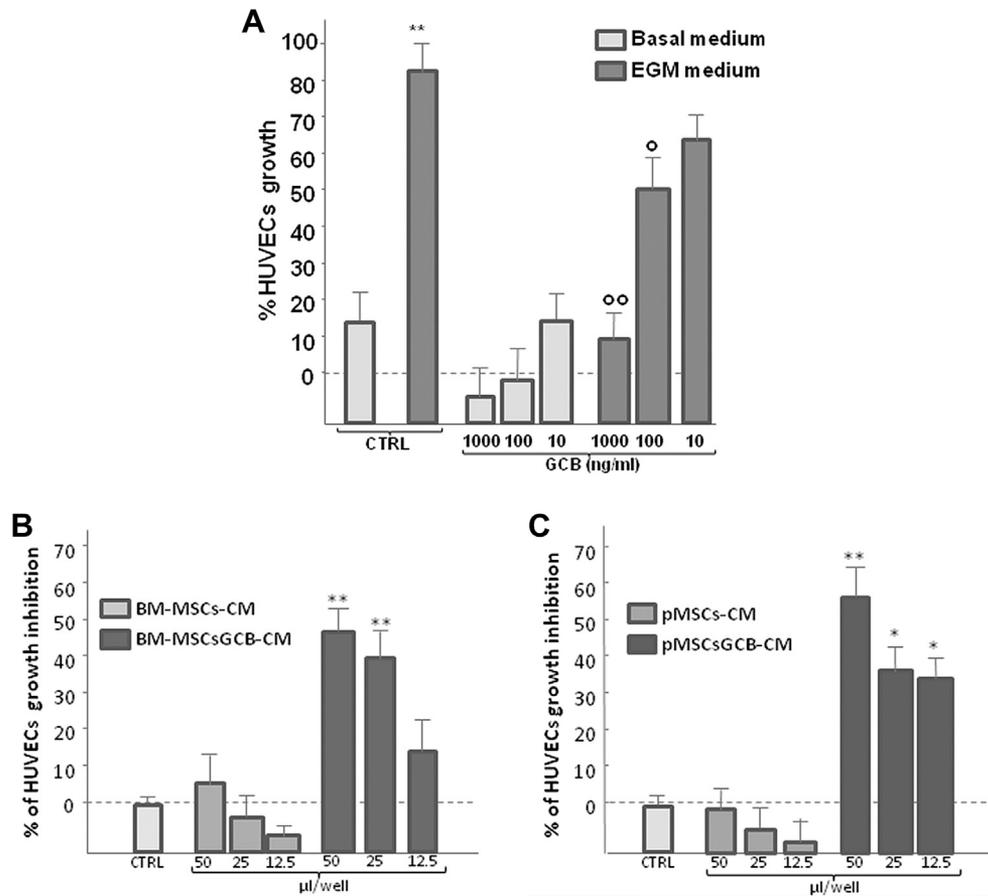
Figure 4. Effect of BM-MSCs and pMSCsGCB-CM on endothelial cell proliferation (A) Inhibitory activity of GCB toward the human endothelial cell line HUVEC. The inhibitory effect was particularly evident when HUVECs were cultured in EGM medium (basal medium + growth supplements). **$P < 0.01$ versus control (CTRL) in basal medium; °$P < 0.05$ and °°$P < 0.01$ versus control in EGM medium. (B, C) Inhibitory activity of conditioned media from, respectively, BM-MSCsGCB and pMSCsGCB toward HUVECs; both of them were able to significantly inhibit endothelial cell proliferation in a dose-dependent manner. *$P < 0.05$ and **$P < 0.01$ versus CTRL (HUVECs grown in EGM medium). Bars in figures are means ± standard deviations of three independent experiments.

concentrations arrest cell-cycle progression in all phases [28]. This situation represents the optimal condition for loading the MSCs with drug; GCB at a concentration of 2000 ng/mL blocks cell division but maintains viability and drug accumulation.

GCB is a pro-drug (a cytidine analogue), internalized by nucleoside transporters. Once internalized, GCB is di- and tri-phosphorylated by deoxycytidine kinase into active metabolites that, respectively, inhibit ribonucleotide reductase, blocking *de novo* DNA synthesis, and incorporate into DNA, making it more difficult to repair [29,30]. No data are reported in the literature about the expression of the concentrative nucleoside transporter 1 (hCNT-1) and its role in MSCs. In our model, we found that both BM-MSCs and pMSCs expressed significant levels of hCNT1, suggesting that this transporter could play some roles in the uptake of GCB by these cells.

MSCsGCB-CM were very active in inhibiting the proliferation of the PDAC cell line, CFPAC-1, whereas the CM of untreated cells did not affect tumor cell proliferation. High-performance liquid chromatography (HPLC) analysis confirmed the presence of GCB in the CM and suggested that the main inactivating mechanism of GCB (catalyzed by deoxycytidine deaminase) is inactive in MSCs [30]. After 48 h of MSC subculture, the drug is not completely released and some GCB remains inside the cells, as demonstrated by the anti-tumor activity of lysates from MSCsGCB and by HPLC analysis.

A single MSC can release 0.076 pg of GCB (mean value between BM-MSCs and pMSCs). This means that 10^6 MSCs, easily achievable, can deliver 76 ng of GCB and that GCB-primed cells injected *in vivo* could release *in situ* (eg, in a typical tumor volume of 1 mL) a drug concentration 100 times higher than the IC_{50} value for CFPAC-1. A preliminary study of co-culture MSCs/TCs performed with the aim to set up follow-on preclinical studies in mice confirmed that the inhibitory effect of MSCsGCB is directly proportional to their numbers.

MSCs produce many factors having autocrine/paracrine functions, and interest in their secretome has increased during recent years because of its potential application in regenerative medicine. Our study was not intended to address this application of MSCs; however, our preliminary investigation showed that very high concentrations of GCB did not modify the secretome of either BM-MSCs or pMSCs treated with GCB, with the only exception being interleukin (IL)12p40, which significantly increased (approximately 200-fold) in BM-MSCsGCB. We do not know the mechanism behind the p40 stimulation in BM-MSCs only. Because IL-12p40 is a subunit shared with other members of the IL-12 family (eg, IL-23), it is plausible that IL-12p40 and not IL-12p70 could be upregulated. Because both IL12 and IL23 have a role in immune modulation and inflammation, it will be interesting to investigate this observation further and its possible role in BM-MSC influence on the tumor micro-environment.

Indeed, the role of MSCs in pancreatic tumors is a complex issue. These tumors are characterized by important quantitative and qualitative effects of a stroma constituted by both resident cells and those that are attracted there from the BM by inflammatory signals. BM-derived cells probably originate from a CD45+ population resident in the BM that migrate to the injured pancreas and give rise to pMSCs [15]. MSCs might support tumor growth by migrating from the BM to blood vessels of pancreatic carcinoma after the hypoxia-induced secretion of several growth factors (GF): platelet-derived (PDGF), epidermal (EGF) and vascular endothelial (VEGF). Once located into tumor, MSCs interact with endothelial cells and favor tumor blood vessel formation through VEGF secretion [31]. MSCs derived from BM appear to regulate epithelial to mesenchymal transition of a pancreatic tumor–initiating cell population and to maintain it [32]. In such a complex picture, we suppose that a possible therapeutic strategy could lie in the use of the same MSCs as a "trojan horse": MSCsGCB could be integrated into the tumor mass and deliver the drug *in situ* at very high concentrations, difficult to obtain by intravenous injection. This could also contribute to decrease severe toxic side effects.

Our *in vitro* study provides an important proof of concept that needs confirmation *in vivo*. As previously demonstrated with MSCs delivering PTX, we think it is possible to develop a cytotherapy with the use of MSCs co-loaded with GCB and PTX to obtain the strongest anti-tumor effect *in vivo*. In fact, as shown by preliminary experiments, MSCs can uptake PTX and GCB *in vitro* during simultaneous exposures (see Supplementary Information), and their CM have an increased anti-tumor activity.

Pre-clinical study is scheduled in our laboratory to demonstrate the efficacy of such a new therapeutic approach that could open new perspectives for treating such an aggressive neoplasia as pCa.

Acknowledgments

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Supplementary data