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Mesenchymal stromal cells loaded with Paclitaxel (PacliMES) a potential new therapeutic approach on mesothelioma

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ABSTRACT

Malignant pleural mesothelioma is an asbestos-related tumor originating in mesothelial cells of the pleura that poorly responds to chemotherapeutic approaches. Adult mesenchymal stromal cells derived either from bone marrow or from adipose tissue may be considered a good model for cell-based therapy, a treatment which has experienced significant interest in recent years. The present study confirms that Paclitaxel is effective on mesothelioma cell proliferation in 2D and 3D *in vitro* cultures, and that 80,000 mesenchymal stromal cells loaded with Paclitaxel inhibit tumor growth at a higher extent than Paclitaxel alone. An *in vivo* approach to treat in situ mesothelioma xenografts using a minimal amount of 10^6 mesenchymal stromal cells loaded with Paclitaxel showed the same efficacy of a systemic administration of 10 mg/kg of Paclitaxel. These data strongly support drug delivery system by mesenchymal stromal cells as a useful approach against many solid tumors. We look with interest at the favourable opinion recently expressed by the Italian Drug Agency on the procedure for the preparation of mesenchymal stromal cells loaded with Paclitaxel in large-scale bioreactor systems and their storage until clinical use. This new Advanced Medicinal Therapy Product, already approved for a Phase I clinical trial on mesothelioma patients, could pave the way for mesenchymal stromal cells use as drug delivery system on other solid tumors for adjuvant therapy associated with surgery and radiotherapy.

1. Introduction

Malignant pleural mesothelioma (MPM) is a fatal asbestos-related malignancy originating in the mesothelial cells of the pleura. Although pleura remains the most common affected area, the disease can arise in other mesothelial cells including the peritoneum, pericardium, tunica vaginalis of testis and ovary [1–3]. Different chemo-therapeutic approaches have been tested in the past, but results have generally been disappointing. Some activity has been reported for anthracyclines alone or in combination with chemotherapy, and a platinum-based doublet containing a third-generation antifolate (Pemetrexed or Raltitrexed) is considered the front-line standard of care. Recently, the combination of

Abbreviations: AMTP, Advanced Medicinal Therapy Product; AT, adipose tissue; CM, conditioned medium; i.p, intraperitoneal; MPM, Malignant pleural mesothelioma; MSCs, Mesenchymal Stem Cells; PMX, Pemetrexed; PTX, Paclitaxel.

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Nivolumab and Ipilimumab, two immune checkpoint inhibitors, has been approved as first line treatment [4].

Adult mesenchymal stromal cells (MSCs) derived either from bone marrow (BM) or from adipose tissue (AT) have been suggested as suitable cell sources for cell-based therapies. Because of their self-renewal, differentiation and paracrine properties, MSCs possess intrinsic therapeutic potential and can be easily manipulated *in vitro* and engineered to produce or deliver therapeutic molecules [5–8]. In our previous reports, we have shown that MSCs can acquire strong anti-cancer activity upon *in vitro* exposure to very high dose of chemotherapeutic agents, and that MSCs loaded with paclitaxel (PTX) strongly inhibit murine B16 melanoma tumor growth *in vivo* upon intra-tumoral or intra-venous injection (i.v.) [9,10].

In our previous papers we reported that human MSCs from different sources, such as bone marrow, adipose tissue, or gingival tissue, are very resistant to PTX direct cytotoxicity (at least until 10,000 ng/ml) and these data allow us to select the right amount of PTX to use for loading these cells for drug delivery, and the release over time [10–12].

Thus, MSCs become effective drug-releasing mesenchymal cells because they can uptake and release the drug and then kill tumor cells when located nearby.

Regarding MPM, a significant tumor-inhibiting effect has been described by conditioned media (CM) from human lung MSCs *in vitro* [13], and our previous studies also demonstrated that both lysates and CM from AT-MSCs inhibited the proliferation of mesothelioma cell lines [14]. Furthermore, our *in vivo* study confirmed that a loco-regional treatment of mesothelioma xenograft with high amounts of AT-MSCs resulted in a dramatic inhibition of tumor growth comparable with that produced by systemic administration of PTX [11]. Previous *in vitro* studies verified that MSCs loaded with PTX are able to release the drug and to inhibit the proliferation of NCI-H28 mesothelioma cell line [15]. In the present study, we show the *in vivo* effect exerted on mesothelioma xenografts by "low amount" of MSCs both untreated and primed with PTX (MSCs/PTX). These results confirmed that "locally injected" MSCs do not stimulate mesothelioma growth but produced "per se" a little anticancer effect. On the contrary, the local injection of MSCs/PTX, exerted a remarkable therapeutic effect by inhibiting the tumor progression at a level comparable to that obtained by PTX injected systemically.

2. Materials and methods

2.1. Cell lines

The human mesothelioma cell line MSTO-211H [16] and BEAS-2B, an immortalized but non-tumorigenic epithelial cell line from human bronchial epithelium, were obtained from ATCC (Manassas, VA, USA), which authenticates the phenotype of cell line on a regular basis.

Cells were cultured in complete medium RPMI 1640 + 10% Foetal Bovine Serum (FBS, Euroclone, Milan, Italy) by 1:5 weekly passages. Based on our previous publication [14] and on the characteristic of MSTO-211H cell derived-xenograft growth and sensitivity to PTX we used this cell model for the *in vitro* and *in vivo* studies.

2.2. Mesenchymal stromal cells (MSCs)

Adipose tissue lipoaspirates from adult donors were collected after written informed consent in accordance with the Declaration of Helsinki. The approval of the collection was obtained from the Institutional Ethical Committee of IRCCS Cà Granda Ospedale Maggiore Policlinico of Milan (#978) and concerned redundant biological material to use for research was otherwise discarded.

Mesenchymal stromal cells were isolated from lipoaspirate as previously described [17]. Briefly, lipoaspirate was treated by enzymatic digestion with type I collagenase (Life technologies, Carlsbad, CA, USA) and after centrifugation (1000xg, 15 min), the floating fraction was discarded, and cellular pellet collected. Cells were plated on 25 cm^2 flask (Euroclone) and expanded in DMEM low glucose medium (Euroclone) supplemented with 5% platelet lysate Stemulate (Cook Regentec, Indianapolis, IN, USA) and 2 mM L-glutamine (Euroclone) by incubation at 37 °C, 5% CO_2. Primary cultures were analysed for their proliferation rate (Population Doubling Time = 30–36 hs), clonogenicity (CFU-F assay = 75%), the expression of the typical mesenchymal stem cell markers (CD90, CD105 and CD73), the absence of the ematopoietic/endothelial markers (CD34, CD45 and CD31) and the differentiation ability towards mesodermal lineages (osteogenic, adipogenic, chondrogenic) as previous described [14].

2.3. Loading MSCs with Paclitaxel

To load MSCs with PTX, we used the methodology of priming MSCs with very high drug dosages according to the procedure previously described [18]. Briefly, subconfluent cultures ($12x10^4$ cells/cm^2) of cells were exposed to 2000 ng/ml PTX (PTX clinical grade, TEVA, Milan, Italy). After 24 h of incubation, the cultures were washed twice with PBS, detached with trypsin, and washed twice in HBSS. MSCs/PTX were then seeded in a new 25 cm^2 flask in DMEM low glucose + 10% FBS and 2 mM L-glutamine.

2.4. In vitro drug efficacy on MSTO-211H cells

The effect of Pemetrexed (PMX, obtained from the inpatient pharmacy of University Hospital of Parma, Italy) and PTX against cell proliferation was studied by a three-day culture in 96 multiwell plates (Sarstedt, Germany) and cell viability evaluated by MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium) as previously described [19].

2.5. Transwell assay

The effect of MSCs and MSCs/PTX on MSTO-211H proliferation was analyzed using transwell inserts. Increasing amounts ($2x10^4$, $4x10^4$, $8x10^4$) of MSCs or MSCs/PTX were seeded in a 24-well plate (diameter 1.9 cm^2), while $3x10^3$ MSTO-211H cells were seeded onto the insert with pore size of 0.4 μm (Falcon-Corning, NY, USA). After 5 days of incubation (37 °C, 5% CO2), cells in the insert were stained with 0.25% crystal violet (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 10 min, washed with PBS buffer and eluted with 0.3 ml of 33% glacial acetic acid. The absorbance of the eluted dye was measured at 550 nm. Alternatively, cells were counted by trypan blu exclusion test. Cell death was assessed by Hoechst 33,342 and propidium iodide dual staining as previously described [14].

2.6. Cytokines secretion by MSCs

Conditioned media (CM) of both MSCs and MSCs/PTX were collected after 6 days of incubation (10^6 cells in 10 ml of a 75 cm^2 flasks). Five different MSC donors were used. Forty cytokines/growth factors were evaluated using "multiplex bead-based xMAP technology" (Bio-Plex Human Cytokine 27-Plex Panel, Biorad Laboratories, Hercules, CA, USA) following manufacturer's instructions.

2.7. Treatment of 3D multicellular cultures of MSTO-211H

3D multicellular tumor spheroids were prepared as described previously [20]. Briefly, cultures were initiated by seeding 2.5×10^5 cells/ml in 13 ml of complete Iscove modified Dulbecco's medium (Euroclone) + 10% FBS (Euroclone) in polycarbonate Erlenmeyer flasks (Euroclone) incubated in a gyratory incubator (Colaver, Milan, Italy) at 60 rev/min at 37 °C in air atmosphere. Homotypical aggregations, visible after 3 days of culture, were usually complete within 5 days. At the sixth day, 3D tumor cell aggregates were exposed to PTX at two

different concentrations of PTX (10 and 20 ng/ml) and kept in the incubator for additional 48 h. Control cultures received the same volume of medium.

2.8. Caspase-3 expression and enzymatic activity

Caspase-3 expression in MSTO-211H 3D aggregates after 48 h of PTX treatment (10 and 20 ng/ml) was evaluated by Real-Time quantitative PCR (RT-q PCR) analysis: total RNA was extracted from pellet of MSTO-211H 3D aggregates not treated (CTRL) or treated with PTX, following instructions of PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA was treated with DNase to exclude DNA contamination and then 1 μg of RNA was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Samples were analyzed using Rotor-Gene Q cycler (Qiagen) and the amplifications were carried out using QuantiNova™ SYBR® Green PCR (Qiagen) in a total volume of 20 μl. The following primer sequences were used (5′-3′): CASPASE-3: FW: ATTTGGAACCAAAGATCATACATGG; REV: TTCCCTGAGGTTTGCTG-CAT. GAPDH: FW: GGAGTCAACG-GATTTGGTCG; REV: CTTCCCGTTCTCAGCCTTGA.

Data were analyzed by using the $2 - \Delta\Delta Ct$ method to obtain the relative expression level, and each sample was normalized by using GAPDH RNA expression. The experiments were carried out in duplicate for each data point.

Enzymatic activity of caspase-3 in spheroids was measured by the Caspase-3 Assay Kit (#ab3940, Abcam, Cambridge, UK). Briefly, the MSTO-211H spheroids were treated with PTX 0 (CTRL), 10, and 20 ng/ml and incubated 48 h. At the end, spheroids were harvested and lysed on ice. After centrifugation, the presence of caspase-3 activity in the lysate was examined by measuring the output of OD 405 nm on a microplate reader, according to the supplier's protocols.

2.9. Wound healing assay

To evaluate the effect of PTX released by MSCs on cell migration, CM from MSCs (CM CTRL) and from PTX-loaded MSCs (CM PTX) were tested in wound healing assay, as previously described [21]. Briefly, MSTO-211H cells were seeded $2x10^5$ cells/well in 24-well plates and grown 24 h until they reached 90% of confluence. A cell-free gap was created on the monolayer by scratching with a pipet tip, then the following treatments were added to each well: DMEM low glucose with 2% FBS in control cells (CTRL), PTX at a final concentration of 20 ng/ml, CM CTRL, and CM PTX. After 24 or 48 h, scratch length was measured by Wound Healing Size Tool of ImageJ software (NIH, MD, USA) and each condition normalised vs. its own T0, to obtain the percentage of wound closure. Each experiment was repeated in triplicate.

2.10. In vivo experiments

The study was performed in a subcutaneous xenograft model in mice as previously described [14]. Briefly, Balb/c-Nude female mice (Charles River Laboratories, Calco, Italy) were subcutaneously injected with 10^6 human MPM MSTO-211H cells. Mice received three weekly treatments as follows: intraperitoneal (i.p.) treatment with 10 mg/kg of free PTX (TEVA) (0.2 mg/mouse); in situ treatment with 10^6 MSCs; in situ treatment with 10^6 MSCs/PTX (equivalent to 0.05 mg/kg or 0.001 mg/mouse of PTX). Mice were treated at days 0, 7, 14, and the tumor growth was evaluated until the 6th day after the last treatment. Tumor xenografts were measured three times per week using a digital caliper and tumor volume was determined using the formula: $(\text{length} \times \text{width}^2)/2$ as previously described [22]. On day 20, mice were euthanized by cervical dislocation and the tumor nodules were collected for further analyses. Moreover, mouse blood was collected by cardiac puncture. All experiments involving animals and their care were performed with the approval of the Local Ethical Committee of University of Parma (Organismo per la Protezione e il Benessere degli Animali, OPBA) and of the Italian Ministry of Health, in accordance with the institutional guidelines that are in compliance with national (D.Lgs.26/2014) and international (Directive 2010/63/EU) laws and policies. As previously described [23], the tumor masses, excised, formalin fixed, and paraffin embedded were processed for histochemical analysis. Briefly, the morphometric evaluation of xenograft composition was performed on Masson's trichrome-stained sections to evaluate neoplastic tissue, fibrosis or necrosis. Macro images of the entire section were captured by an optical microscope (DMD108 Leica, Wetzlar, Germany) with integrated digital camera (200X final magnification). Plasmatic levels of PTX were measured by HPLC.

2.11. Statistical analysis

Data are expressed as the mean ± standard error (SEM). For statistics, one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test (to adjust for pairs comparisons) were performed by using GraphPad software (GraphPad Software Inc., San Diego, CA, USA). p values < 0.05 were considered statistically significant.

3. Results

3.1. Sensitivity of MSTO-211H cells to Paclitaxel in vitro

Sensitivity of mesothelioma MSTO-211H cells to PTX was assessed in a 3-day anti-proliferative test in comparison with Pemetrexed (PMX) (Fig. 1A) according to the MTT assay. Both PMX and PTX produced a significant ($p < 0.001$) dose-dependent inhibition with greater activity exerted by PTX. IC_{50} values of the two drugs were 3.82 ± 0.16 ng/ml for PTX and 10.22 ± 1.63 ng/ml for PMX. The cytotoxic activity of PTX evaluated on MSTO-211H 2D cultures was also confirmed in 3D multicellular cultures (Fig. 1B), where aggregates had difficulty forming in the presence of the drug. Moreover, a significant 1.8- and 2.5-fold increase of caspase-3 transcript was observed by RT-q PCR upon treatment with 10 and 20 ng/ml PTX compared to the control sample, suggesting an activation of the apoptotic pathway (Fig. 1C). Caspase-3 enzymatic activity was also detected on 3D tumor aggregates, pointing out a 20 and 40% increase after treatment with 10 and 20 ng/ml of PTX (Fig. 1D).

3.2. Efficacy of MSCs/PTX on MSTO-211H cell proliferation, cell death and cell migration in vitro

In vitro efficacy of MSCs and of MSCs/PTX on MSTO-211H cell growth has been evaluated in a transwell assay with increasing number of MSC or MSCs/PTX cells: 20,000, 40,000, and 80,000. Both 20,000 and 40,000 MSCs and MSCs/PTX produced an identical significant ($p < 0.01$) inhibition rate of the tumor cell growth, compared to the control sample. Interestingly, 80,000 MSCs/PTX were able to produce an inhibition higher than that observed with unprimed MSCs, and this difference was statistically significant ($p < 0.01$) (Fig. 2). This is in agreement with our previous results [14] reporting that the MSCs secretome is "per se" able to inhibit MSTO-211H cells also in the absence of cell-to-cell contact. However, an 80,000 MSCs/PTX were able to release enough PTX to enhance the MSCs basal effect and give an inhibition rate of MSTO-211H proliferation higher than that obtained with MSCs alone and also with 4.2 ng/ml of the pure drug ($p < 0.05$).

To better understand the anti-proliferative efficacy of MSCs/PTX compared to MSCs cells, we evaluated cell viability by cell counting and cell death analysis. MSTO-211H were exposed to 80,000 MSCs or MSCs/PTX; as shown in Fig. 2B MSCs decreased cell proliferation and this inhibition was further enhanced when MSC were loaded with PTX. This reduction of cell viability was associated with an increased in cell death.

The effects of MSCs/PTX and MSCs were also evaluated on BEAS-2B cells, a non-tumorigenic human cell line established from normal human bronchial epithelium. As shown in Fig. 2C, BEAS cell proliferation was affected either by MSCs and MSCs/PTX, even if to a lesser extent than

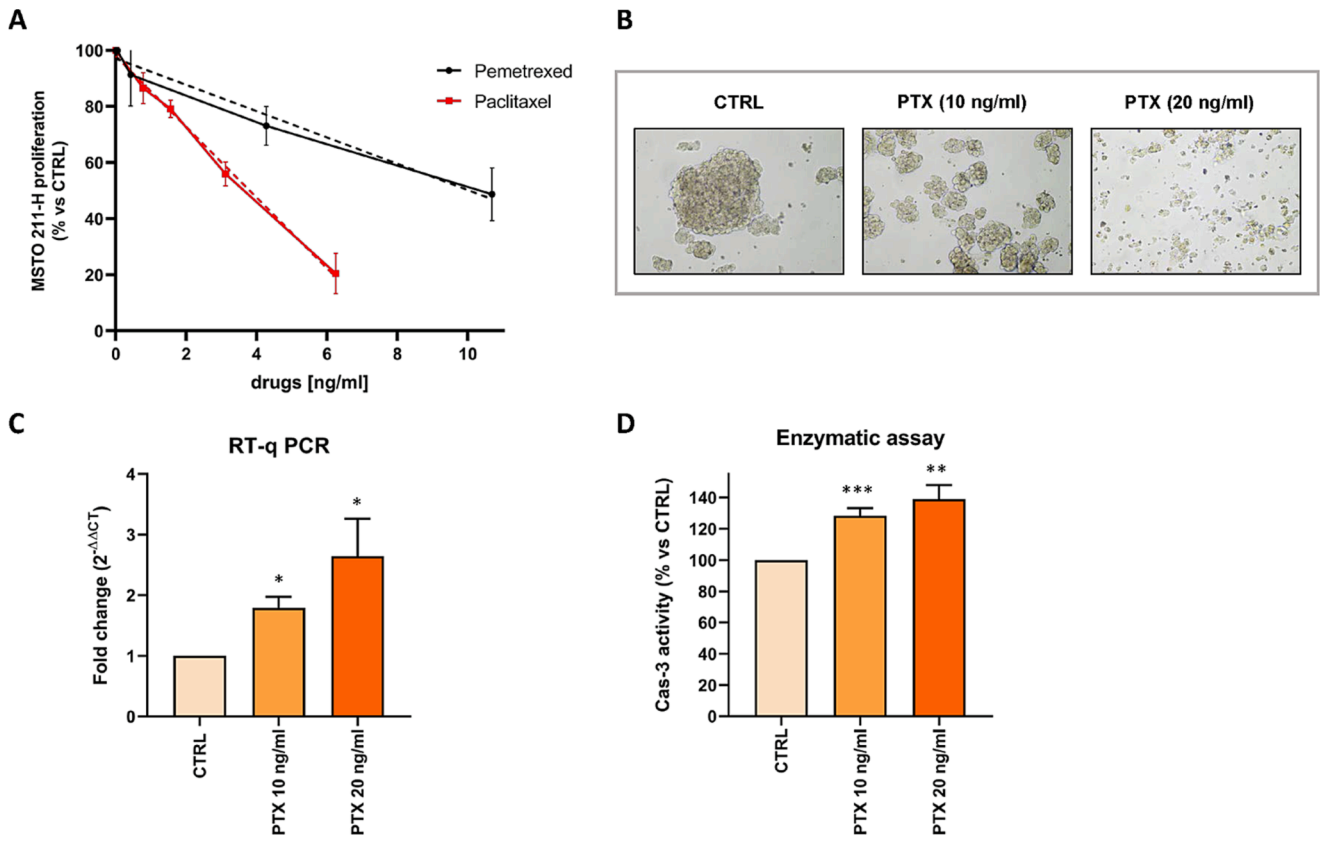

Fig. 1. ***In vitro*** **antitumoral activity of Paclitaxel (PTX) and Pemetrexed (PMX) on mesothelioma cell line MSTO-211H.** A: The effect of increasing concentrations of PTX and PMX was evaluated by a 3-day antiproliferation MTT assay. The effect was expressed as a percentage of the optical density measured in cultures that did not receive drugs (100% proliferation). Data are the means ± standard error (SEM) of three independent replicates. B: Representative images of MSTO-211H 3D spheroids without treatment (CTRL) or treated 48 h with PTX (10 and 20 ng/ml). C: Quantitative Real Time PCR of proapoptotic molecule Caspase-3. D: Enzymatic detection of Caspase-3 activity. Values are the mean of three replicates ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001).

MSTO-211H cells, and no cell death was detected.

To prove the anti-migratory activity of PTX released by MSCs, a wound healing assay on MSTO-211H cells upon 24 and 48 h of treatment was performed in the presence of CM from MSCs or MSCs/PTX cultures. As shown in Fig. 3, cells showed a significant high percentage of wound closure when exposed to CM from MSCs culture, whereas the treatment with CM form MSCs/PTX CM slowed down the wound healing starting from 24 h.

3.3. Cytokines production by MSCs and MSCs/PTX

The analysis of MSCs secretome before and after their loading with PTX was performed on conditioned media of 9 donors by a qualitative/quantitative measure of 40 cytokines. As expected, among the donors a significant ($p < 0.02$) difference of cytokine/growth factors production was observed (from undetectable low levels, up to 30,000 pg/mL). In our study, we considered a cut-off of 2000 pg/ml for cytokines production and therefore only eight molecules were analysed (Fig. 4). Only few differences in the cytokines/growth factor secretion were found in MSCs after PTX loading that however did not result statistically significant ($p > 0.05$). Also, the secretion of cytokines recognized as important pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-4, IL-10, IFN-γ) factors did not show any significant differences between MSCs and MSCs/PTX, indicating that the presence of PTX did not modulate their production.

3.4. In vivo efficacy of MSCs/PTX on mesothelioma growth

The effect of MSCs primed with PTX was evaluated *in vivo* on mesothelioma xenografts in mice, measuring volumes of growing tumor masses. Fig. 5A shows data of tumor growth in the presence of MSCs alone, i.p. of PTX, MSCs/PTX, and in the absence of treatments (CTRL). Results clearly indicate that in situ treatment with MSCs/PTX produced the same inhibitory effect on tumor growth as observed by treating mice with free PTX administered intraperitoneally. At day 14, when mice received the third and last treatment, a significant ($p < 0.05$) lower volume of tumors was measured with PTX and MSCs/PTX, whereas a trend of inhibition, not significant, was observed in mice treated with unprimed MSCs. After the last treatment (day 14) tumor volumes were monitored for further 6 days, and as shown in Fig. 5C, the tumor progression in mice treated with MSCs/PTX was significantly ($p < 0.05$) lower than that observed in mice treated with PTX or MSCs. This result appears to be most clearly evidenced by expressing the tumor growth as increasing tumor volume (ΔV = volume at day 20 – volume at day 14) (Fig. 5D) after treatment stopping. Histological analysis was performed on the tumor masses excised on day 21. The hematoxylin-eosin preparation did not show significant differences. In addition, the morphometric evaluation to quantify the percentage of tissue occupied by the neoplastic, fibrotic, and necrotic components in the different treatments did not indicate significant differences among the fractions (Fig. 5E-F).

4. Discussion

Our preliminary *in vitro* study confirmed that PTX is effective on MSTO-211H mesothelioma cell proliferation and exerts an activity higher than that of Pemetrexed (Fig. 1A). The cytotoxic activity of PTX on MSTO-211H has been confirmed also in 3D multicellular cultures (Fig. 1B) and is associated with apoptosis induction. Although

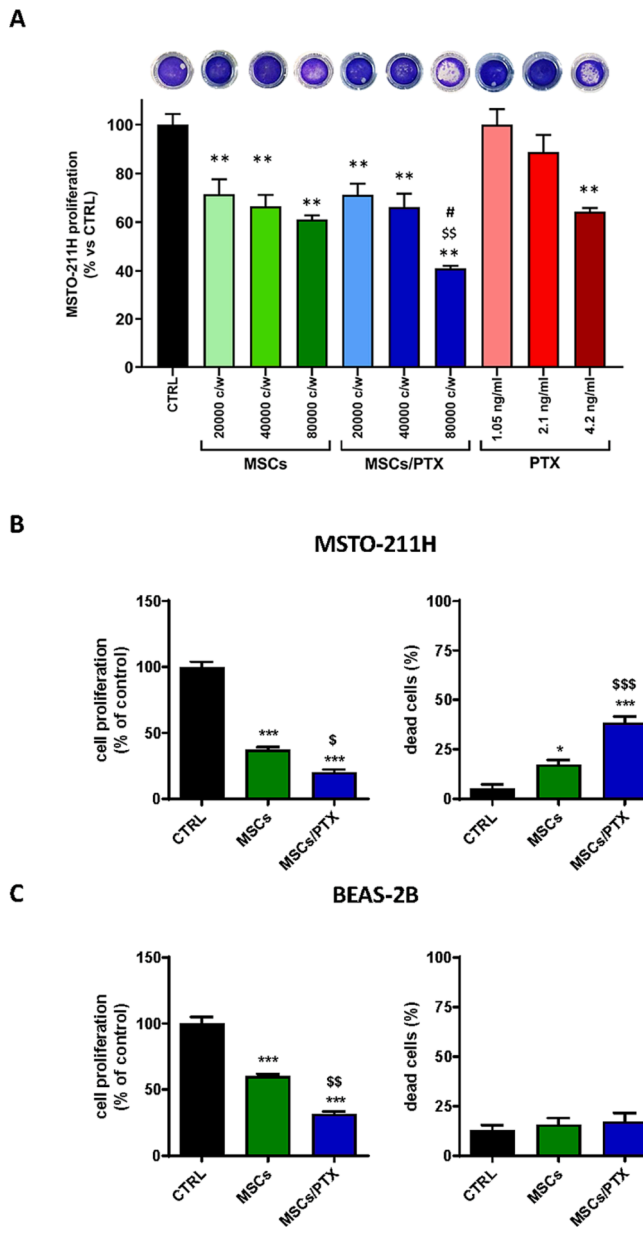

Fig. 2. Effects of MSCs and MSCs/PTX on MSTO-211H and BEAS-2B proliferation and cell death in a 2D co-culture assay. A) The histogram reports the MSTO-211H cells proliferation in transwell inserts after 5 days of co-culture with MSCs, MSCs/PTX (20,000, 40,000, 80,000 cells/well) or treated with PTX (1.05, 2.1, and 4.2 ng/ml). Cell proliferation was evaluated by crystal violet assay. Over the columns, representative images of transwell inserts with MSTO-211H cells after staining with crystal violet were reported. B) After 72 h of co-colture with 80,000 MSCs or MSCs/PTX proliferation of MSTO-211H was evaluated by counting the cells in a Bürker hemocytometer by trypan blue exclusion and cell death was quantitated by fluorescence microscopy analysis on Hoechst 33342 and propidium iodide-stained cells. C) BEAS-2B cells were co-cultured with 80,000 MSCs or MSCs/PTX for 72 h; then cell proliferation and cell death were evaluate as described above. Data are the mean ± SEM of three independent replicates. Statistical analysis was performed using one-way ANOVA. (* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$ vs CTRL; # $p < 0.05$ vs 4.2 ng/ml PTX; $ $p < 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$ vs 80.000 MSCs).

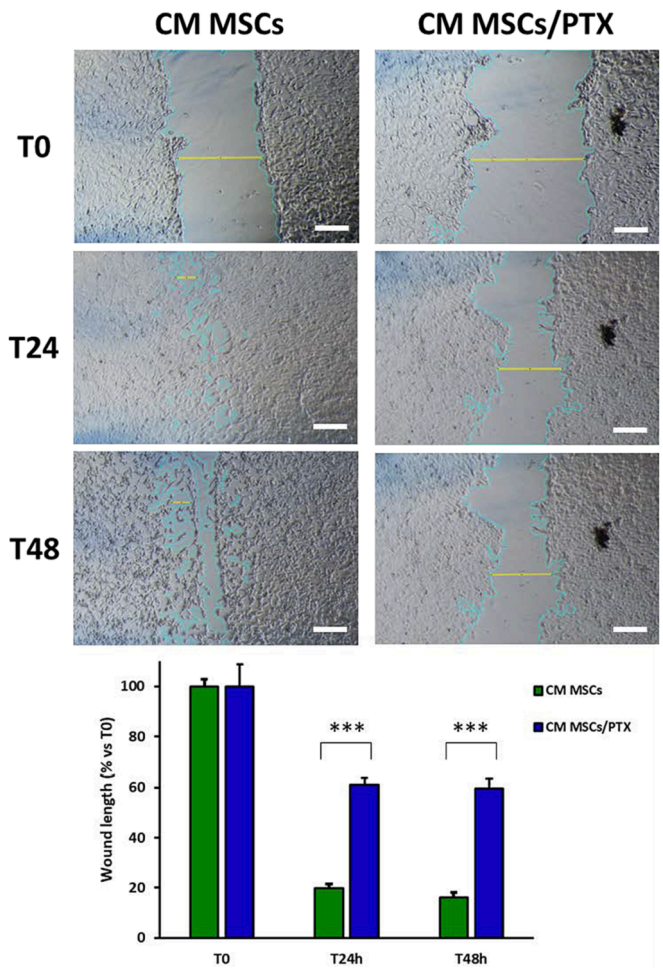

Fig. 3. Inhibitory effect of CM from MSCs-PTX on cell migration. Wound healing assay was performed on MSTO-211H cells treated with CM derived from MSCs or CM derived from MSCs-PTX. MSTO-211H cells were seeded and incubated 24 h, then a wound healing was performed in each well. A picture of the same wound region was taken upon 0, 24 and 48 h of treatment for each sample. Images were analysed by Wound Healing Size Tool of ImageJ software (scale bar: 200 μm) and the scratch length was measured for each image. Data were reported in graphs as a percentage value normalised to its own T_0 and each point represents the mean ± SEM of at least 3 measurements. One-way ANOVA was used to determine p values (*** $p < 0.0001$).

longstanding studies did not indicate PTX as a first line drug in mesothelioma [24], we must consider that they could have underestimated its clinical potential. In fact, as suggested by Giordano and colleagues, Paclitaxel cannot be excluded for clinical purpose, because poor quality results could be due to the heterogeneous distribution of this high lipophilic drug [25]. Of course, this means that different formulations or delivery systems could significantly modify its efficacy.

Based on our previous study on the inhibitory effect on MSTO-211H of the MSC secretoma [14], in this report we investigated co-culture of MSCs and MSTO-211H in a transwell assay. Our preliminary observations were confirmed, since we found that MSCs can "per se" inhibit mesothelioma cell proliferation *in vitro*, but we also evidenced that if MSCs are loaded with PTX their efficacy was increased. Thanks to the release of the drug in amount able to significantly affect the MSTO-211H proliferation, 80,000 MSCs primed with PTX were able to give a 50% inhibition rate of tumor cells growth, higher than that obtained by treating tumor with 4,2 ng/ml of pure Paclitaxel. Moreover, MSCs/PTX enhanced cell death induction when compared to MSCs in MSTO-211H but did not exert cytotoxic effect in BEAS-2B cells (Fig. 2). Based on these observations, the *in vivo* study was designed by using a minimal amount of MSCs/PTX to treat in situ mesothelioma xenografts in mice in comparison with both unprimed MSCs and the administration of pure PTX. As expected, the different treatments (Fig. 5) were not able to completely inhibit tumor growth progression and the tumor volume also increased after intraperitoneal treatment with PTX, confirming the high

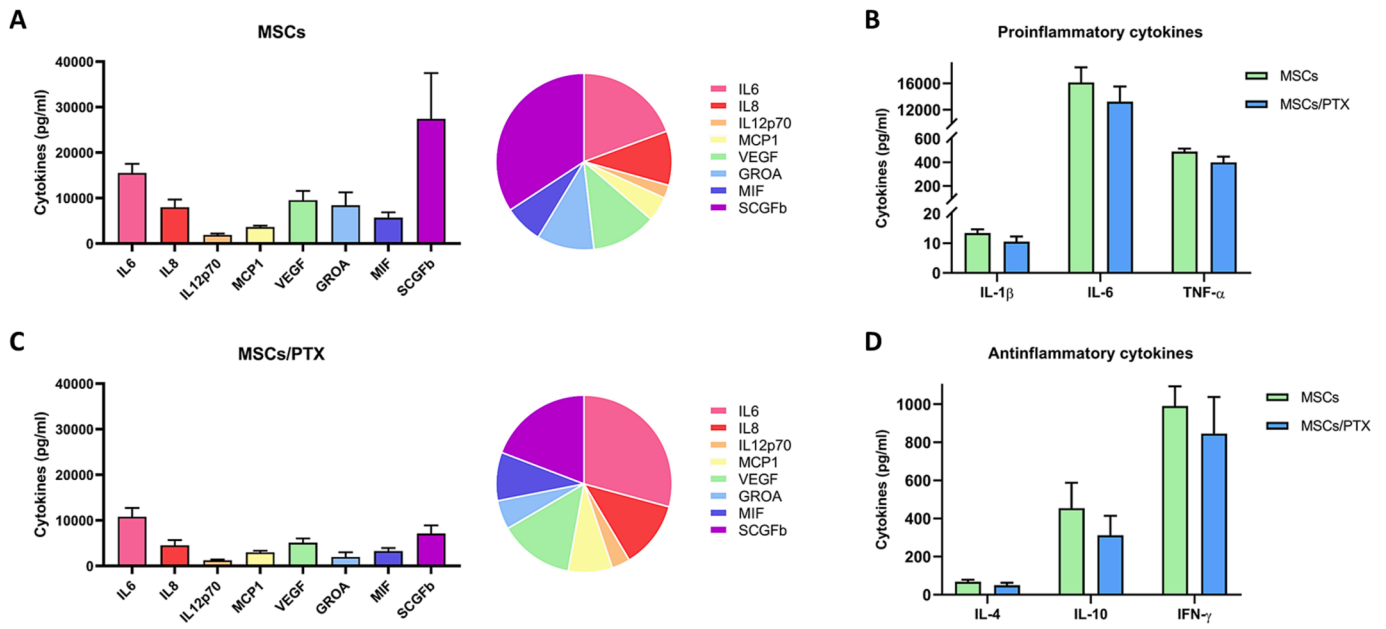

Fig. 4. MSCs and MSCs/PTX secretome analysis. Histograms report eight cytokines/growth factors measured in MSCs (A) and MSCs/PTX (C) conditioned media (expressed in pg/ml). Each point represents the mean ± SEM of the determinations performed on nine different MSCs donors. Proinflammatory and anti-inflammatory cytokines in MSCs and MSCs/PTX secretome were also evaluated in MSCs (B) and MSCs/PTX (D) conditioned media (expressed in pg/ml). Each point represents the mean ± SEM of the determinations performed on nine different MSCs donors.

aggressiveness of such type of cancer. Unfortunately, our experimental design did not allow to evaluate the animal survival, since mice were sacrificed when the tumor masses reached a specific volume indicated by the Italian recommendations on animal welfare. Despite these limitations, our results suggest some important considerations to drive further investigations. Our study confirms that a relatively low amount of adipose tissue derived MSCs (10^6 cells corresponding to $5x10^7$ MSCs/kg) exert a certain inhibition of tumor progression (Fig. 5). This is an important message underlining that MSCs do not promote the growth of subcutaneous xenografts of mesothelioma and contradicts the point of view that MSCs could participate to the stimulation of this type of tumor due to the similar mesenchymal origin. The weak trend of inhibition is in agreement with our previous report that clearly demonstrated that higher amounts of MSCs alone ($5x10^6$ cells corresponding to $2.5x10^8$ MSCs/kg) were effective in producing a significant inhibition of mesothelioma models [14].

A further important result is that the same amount of MSCs (10^6 cells) primed with Paclitaxel (MSCs/PTX) were able to produce a remarkable inhibition of the tumor growth that was comparable to that obtained by the i.p. administration of PTX (Fig. 5). Based on previous experiences, we know that MSCs/PTX, in our standardized conditions, can incorporate up to 1 pg/cell of drug [26]. Therefore, we could assume that the maximum drug delivery potential of 10^6 MSCs/PTX could be close to 1 μg of drug (equivalent to 50 μg/kg). This means that such an amount of MSCs/PTX, injected locally, have the same tumor inhibition rate obtained with the systemic administration of 10 mg/kg of drug, a dosage that is 200 times higher. This high efficacy of PTX released by MSCs is only partially surprising because the MSCs/PTX are injected in situ, while systemic treatment needs higher dosages of drug to reach the same toxicity. Moreover, MSCs/PTX efficacy is the consequence of a slow and long-lasting release of the drug linked to exosomes/microvesicles and only partially in a free form, increasing its bioavailability [27–30]. Such a type of drug injection, very close to the tumor area, can mimic a liposome-mediated drug delivery system, increasing its pharmacological efficacy on cancer cells.

As previously demonstrated, loco-regional treatment with microfragmented fat loaded with PTX could significantly contribute to reduce the drug toxicity if compared with systemic administration [31]. To support this assumptions, in our study we evaluated in three mice the plasmatic level of PTX two hours following loco-regional treatment with MSCs/PTX and found mean value of 16.6 ± 2.53 nM versus a plasmatic concentration of 779 ± 249.7 nM after systemic injection of PTX. This is predictive of a lower toxic effect also in the presence of anticancer efficacy. It is also known that a local treatment with the drug in a free form would not have the same local effect because it would quickly disperse into the circulation with poor local efficacy.

The immunocompromised model allows to directly relate the inhibition of human tumor growth to the specific therapeutic treatment with human MSCs or MSCsPTX independently from immune response. It was difficult to evaluate the effect of our new treatment independently from a possible immunological mechanism against cancer using immunocompetent mice although in nude mice some level of immunocompetence is maintained. Of course, it would certainly be very interesting to also evaluate the role of the immune response in a syngeneic mouse model or in an induced cancer model. This is possible due to the low immunogenicity of MSCs with a low risk of allogeneic immune rejection [32].

In conclusion, our data strongly suggest that drug delivery by MSCs can be a very useful approach in the treatment of many solid tumors and therefore we look with great interest the favorable opinion recently expressed by the Italian Drug Agency on the procedure of loading MSCs with PTX in a large-scale bioreactor system and their storage until clinical use [26]. Furthermore, this new Advanced Medicinal Therapy Product (AMTP) has been approved for a Phase I clinical trial on patients bearing mesotheliomas and we hope that it could pave the way to adopt "PacliMES" (Mesenchymal Stem Cells loaded with Paclitaxel) as adjuvant therapy against other solid tumors in association with surgical procedures and radiotherapy. It should not be underestimated that this procedure could have production costs comparable than those of current anticancer chemotherapy.

CRediT authorship contribution statement

Valentina Coccè: Data curation, Investigation, Methodology, Writing – review & editing. **Mara Bonelli:** Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Silvia**

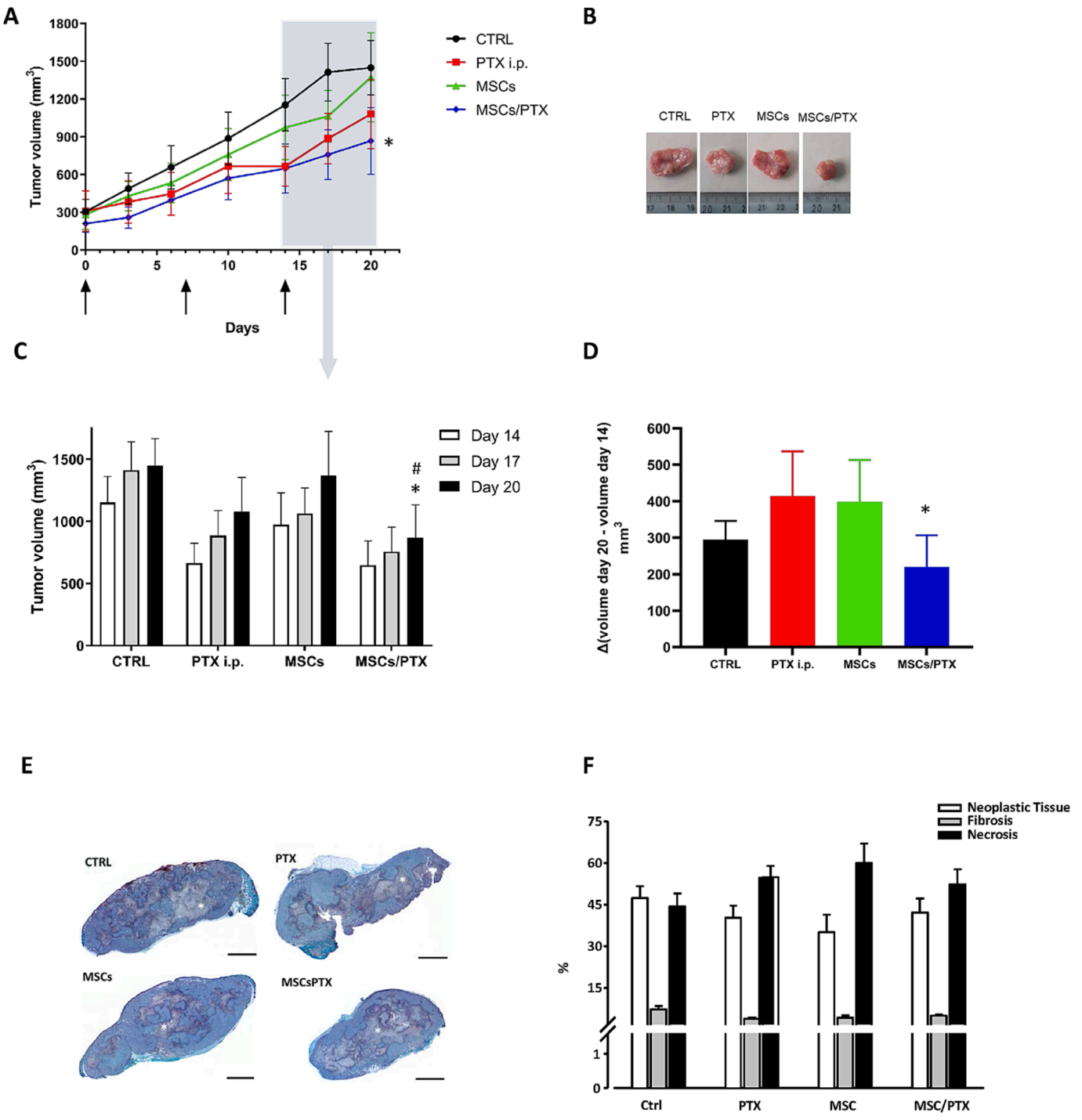

Fig. 5. Effects of MSCs and MSCs/PTX on MSTO-211H xenograft model. MSTO-211H cells were subcutaneously inoculated into BALB/C nude mice, and once tumors had reached an average size of approximately 100 mm^3, animals were treated once a week (at days 0, 7 and 14; black arrows) with vehicle alone (CTRL), PTX (10 mg/kg), 10^6 MSCs, and 10^6 MSCs/PTX. A: Tumor volumes were measured twice per week and data are expressed as means ± SEM. B: Representative images of dissected xenograft tumors. (C) The tumor volumes expressed in mm^3 (Y axis) on day 14, 17, 20, corresponding to 0, 3, and 6 days after the last treatment, compared to the untreated control animals. Values are expressed as means ± SEM. (D) Values of increasing tumor volume (ΔV = volume at day 20 – volume at day 14) after stop of treatments. Statistical analysis was performed using one-way ANOVA (* $p < 0.05$). (E) Morphometric analysis of tumor xenografts. Macrophotographs of Masson's Trichrome stained sections of subcutaneous MSTO-211H-induced tumor nodules from untreated (CTRL), PTX-treated, MSCs, (MSCs-PTX) injected animals in which collagen deposition (greenish) between neoplastic cells (purple) is apparent. White asterisks indicate necrotic areas. Scale bars = 2 mm. (F) Bar graphs showing the quantitative evaluation of tissue composition (neoplastic tissue, fibrosis and necrosis).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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