A new potential therapeutic approach on mesothelioma with Mesenchymal Stromal Cells loaded with Paclitaxel (PacliMES)

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Eleonora Martegani[1], Valentina Coccè[1], Mara Bonelli[2], Silvia La Monica[2], Roberta Alfieri[2], Luisa Doneda[1], Giulio Alessandri[1], Costanza Annamaria Lagrasta[2], Aldo Giannì[1,3], Valeria Sordi[4], Francesco Petrella[1,5,6], Costantino Corradini[7], Angelo Guido Corsico[8], Giulia Maria Stella[9], Leda Roncoroni[1], Francesca Paino[1] and Augusto Pessina[1]

[1] CRC StaMeTec, Department of Biomedical, Surgical and Dental Sciences, University of Milan, 20122 Milan, Italy. [2] Department of Medicine and Surgery, University of Parma, 43126 Parma, Italy. [3] Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, 20122 Milan, Italy. [4] Diabetes Research Institute, IRCCS San Raffaele Hospital, 20132, Milan Italy. [5] Department of Thoracic Surgery, IRCCS European Institute of Oncology, 20139 Milan, Italy. [6] Department of Oncology and Hemato-Oncology, University of Milan, 20122 Milan, Italy. [7] Department of Biomedical Surgical and Dental Sciences, Sports Trauma Researches Center, State University of Milan c/o 1st Division of Orthopedics and Traumatology, Orthopedic Center Pini CTO - ASST Gaetano Pini, Milan, Italy. [8] Department of Internal Medicine and Medical Therapeutics, University of Pavia Medical School, 27100 Pavia, Italy. [9] Cardiothoracic and Vascular Department, Unit of Respiratory Diseases, IRCCS Policlinico San Matteo, 27100 Pavia, Italy.

Background

Malignant Pleural Mesothelioma (MPM) is a fatal asbestos-related disease, mainly regarding mesothelial cells of the pleura, but also other regions including peritoneum, pericardium, tunica vaginalis of testis and ovary [1–3].

Although MPM is an extremely difficult malignancy to treat, current chemo-therapeutic approaches include anthracyclines, a platinum-based doublet containing a third-generation antifolate (Pemetrexed or Raltitrexed), and the combination of Nivolumab and Ipilimumab, two immune checkpoint inhibitors [4].

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].

We have previously shown that **Paclitaxel (PTX)** is efficiently loaded by MSCs with relative low cytotoxicity (at least until 10,000 ng/ml) and secreted as active chemotherapeutic agent against B16 melanoma tumor upon *in vivo* intra-tumoral or intra-venous injection [9,10].

Thus, MSCs become an effective **drug-delivery system** since they can uptake and release the drug over time and kill tumoral cells when located nearby.

PTX-loaded MSCs showed a significant tumor-inhibiting effect on mesothelioma cell lines *in vitro* since an antiproliferative activity was described by conditioned media (CM) from human lung MSCs [11], and CM and cell lysates from AT-MSCs [12]. 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 [13].

In this study, we show *in vitro* activity of PTX on mesothelioma 3D spheroids and the activity of MSCs/PTX on mesothelioma proliferation and migration. Moreover, we evaluate the production of cytokines by MSCs and MSCs/PTX and finally the *in vivo* effect exerted on mesothelioma xenografts by "low amount" of MSCs both untreated and primed with PTX was highlighted.

Materials and methods

Cell lines. Human mesothelioma cell line MSTO-211H; Mesenchymal Stromal Cells (MSCs) from adipose tissue lipoaspirates extracted from adult donors and loaded with Paclitaxel by a standard methodology previously described [14].

3D spheroids of MSTO-211H. 3D multicellular tumor spheroids were exposed to PTX at two different concentrations of PTX (10 and 20 ng/ml) and kept in the incubator for additional 48h. Then, Caspase-3 gene expression was evaluated by Real-Time quantitative PCR (RT-q PCR) analysis, and data analysed by the $2^{-\Delta\Delta Ct}$ method to obtain the relative expression level, and each sample was normalized by using GAPDH RNA expression. Moreover, enzymatic activity of caspase-3 in spheroids was measured by the Caspase-3 Assay Kit.

Transwell assay. The effect of MSCs and MSCs/PTX on MSTO-211H proliferation was analysed by co-colture using transwell inserts (pore size 0,4 µm). After 5 days of incubation (37 ◦C, 5% CO2), tumoral cell were stained with 0.25% crystal violet for 10 min, washed and eluted with 33% glacial acetic acid. The absorbance of the eluted dye was measured at 550 nm [12].

Cytokines secretion. Conditioned media (CM) of both MSCs and MSCs/PTX were collected after 6 days of incubation (five MSCs donors) and forty cytokines/growth factors were evaluated using "multiplex bead-based xMAP technology".

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 measuring scratch length by Wound Healing Size Tool of ImageJ software.

***In vivo* studies.** The study was performed in a subcutaneous xenograft model in Balb/c-Nude female mice [12] that 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). Tumor xenografts were measured three times per week and on day 20, mice were euthanized. 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 follow national (D.Lgs.26/2014) and international (Directive 2010/63/EU) laws and policies.

Results

Effect of PTX on MSTO-211H 3D spheroids

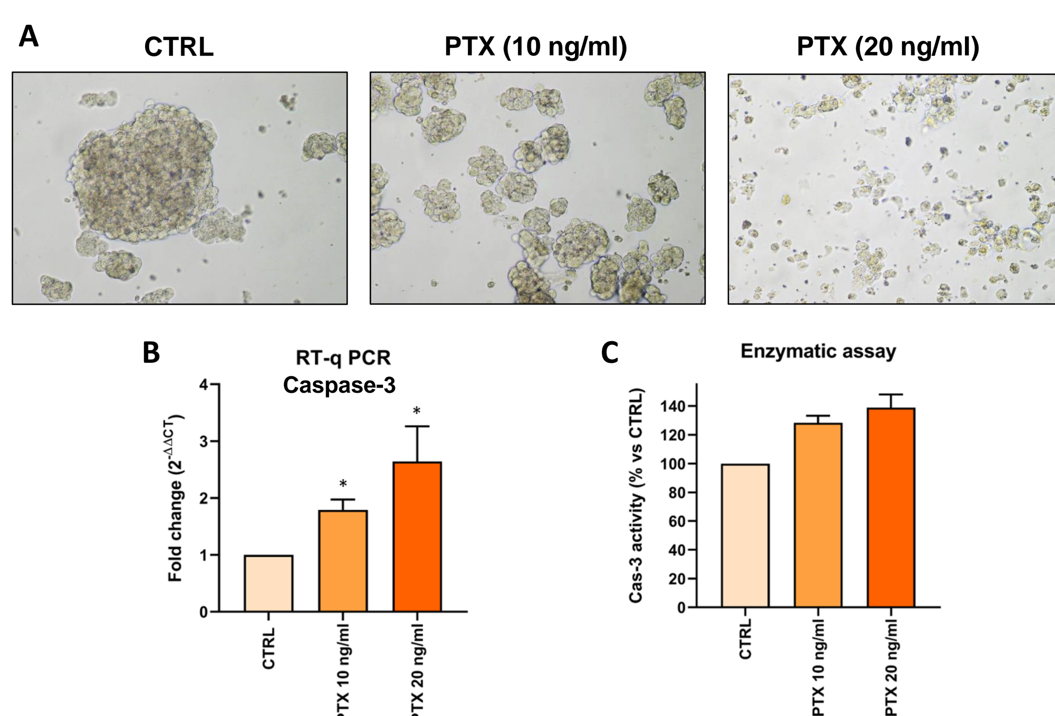

Figure 1. A. Representative images of MSTO-211H 3D spheroids without treatment (CTRL) or treated 48 h with PTX (10 and 20 ng/ml). **B.** Quantitative Real Time PCR of proapoptotic molecule Caspase-3. **C.** Enzymatic detection of Caspase-3 activity. Values are the mean of three replicates ± SEM (*p < 0.05).

Sensitivity of mesothelioma MSTO-211H 3D multicellular cultures to PTX was assessed, where aggregates had difficulty forming in the presence of the drug (Fig. 1A). 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. 1B). 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. 1C).

Effect of MSCs/PTX on MSTO-211H

Figure 2. A. After 72h of co-colture with 80000 MSCs or MSCs/PTX, proliferation of MSTO-211H was evaluated by counting the cells in a Bürker hemocytometer by trypan blue exclusion. **B.** MSTO-211H cells proliferation in transwell inserts after 5 days of co-culture with MSCs, MSCs/PTX (20000, 40000, 80000 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. **C.** Wound healing assay performed on MSTO-211H cells treated with CM derived from MSCs or MSCs-PTX. The graph reports length measure vs T0. (*p< 0.05, **p<0.01, ***p< 0.001).

Anti-proliferative efficacy of MSCs/PTX compared to MSCs cells was evaluated by cell viability (Fig. 2A). MSTO-211H were exposed to 80,000 MSCs or MSCs/PTX and cell viability decreased significantly in both cases but was further enhanced when MSC were loaded with PTX. This agrees with our previous results [12] reporting that the MSCs secretome is "per se" able to inhibit MSTO-211H cells also in the absence of cell-to-cell contact.

In vitro efficacy of MSCs and MSCs/PTX on MSTO-211H cell growth was evaluated by crystal violet staining in a transwell assay with increasing number of MSC or MSCs/PTX cells (20,000, 40,000, and 80,000) and the free drug. 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. 2B). 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 with 4.2 ng/ml of the pure drug (p<0.05).

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. 2C, 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 24h.

Cytokines production by MSCs and MSCs/PTX

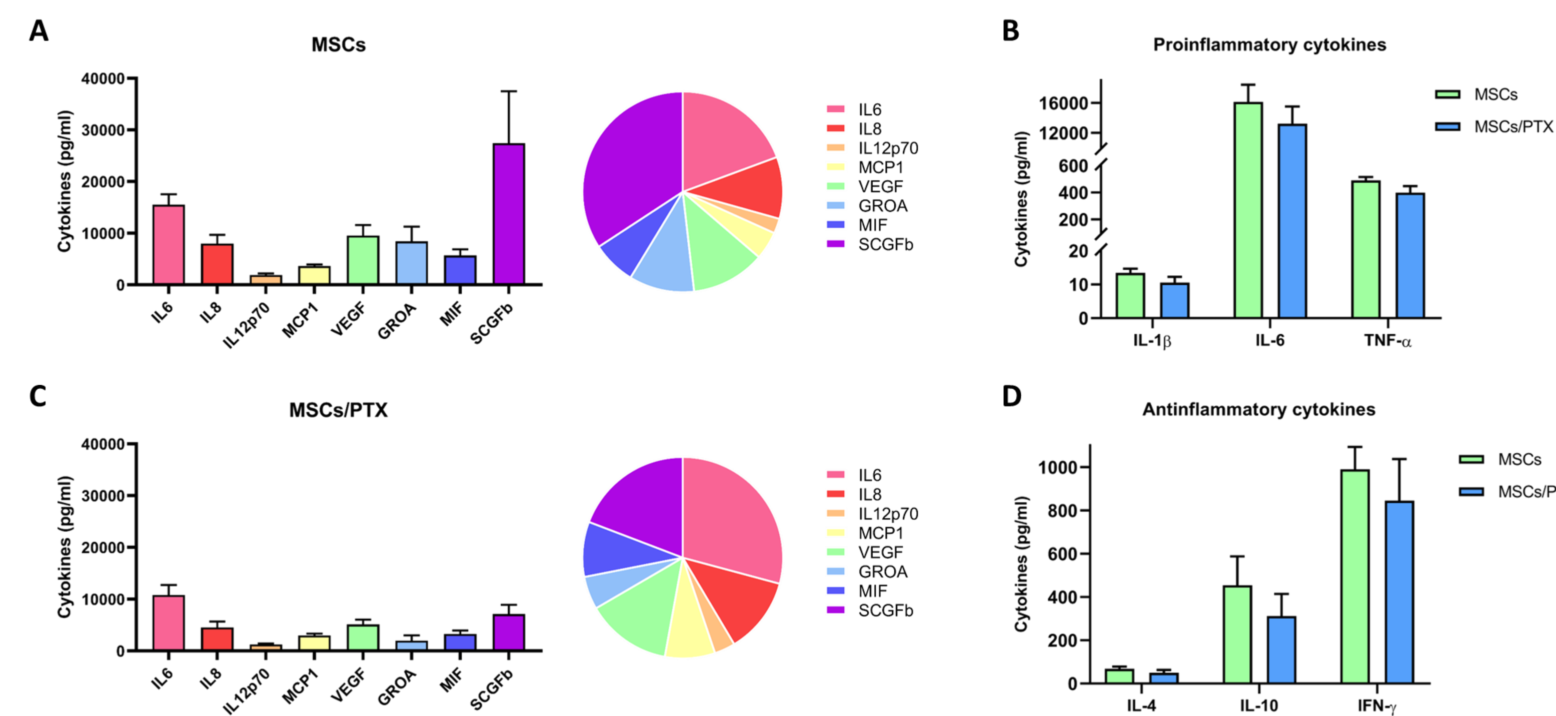

Figure 3. MSCs and MSCs/PTX secretome analysis. Histograms report eight cytokines/growth factors measured in MSCs (**A**) and MSCs/PTX (**C**) conditioned media (expressed in pg/ml). Each point represents the mean ± SEM of the determinations performed on nine different MSCs donors. Proinflammatory and antiinflammatory 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.

The analysis of MSCs secretome before and after PTX loading was performed on conditioned media of 9 donors by a qualitative/quantitative measure of 40 cytokines. Here, we considered a cut-off of 2000 pg/ml for cytokines production and therefore only eight molecules were analysed (Fig. 3). 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). Moreover, 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.

In vivo efficacy of MSCs/PTX on mesothelioma

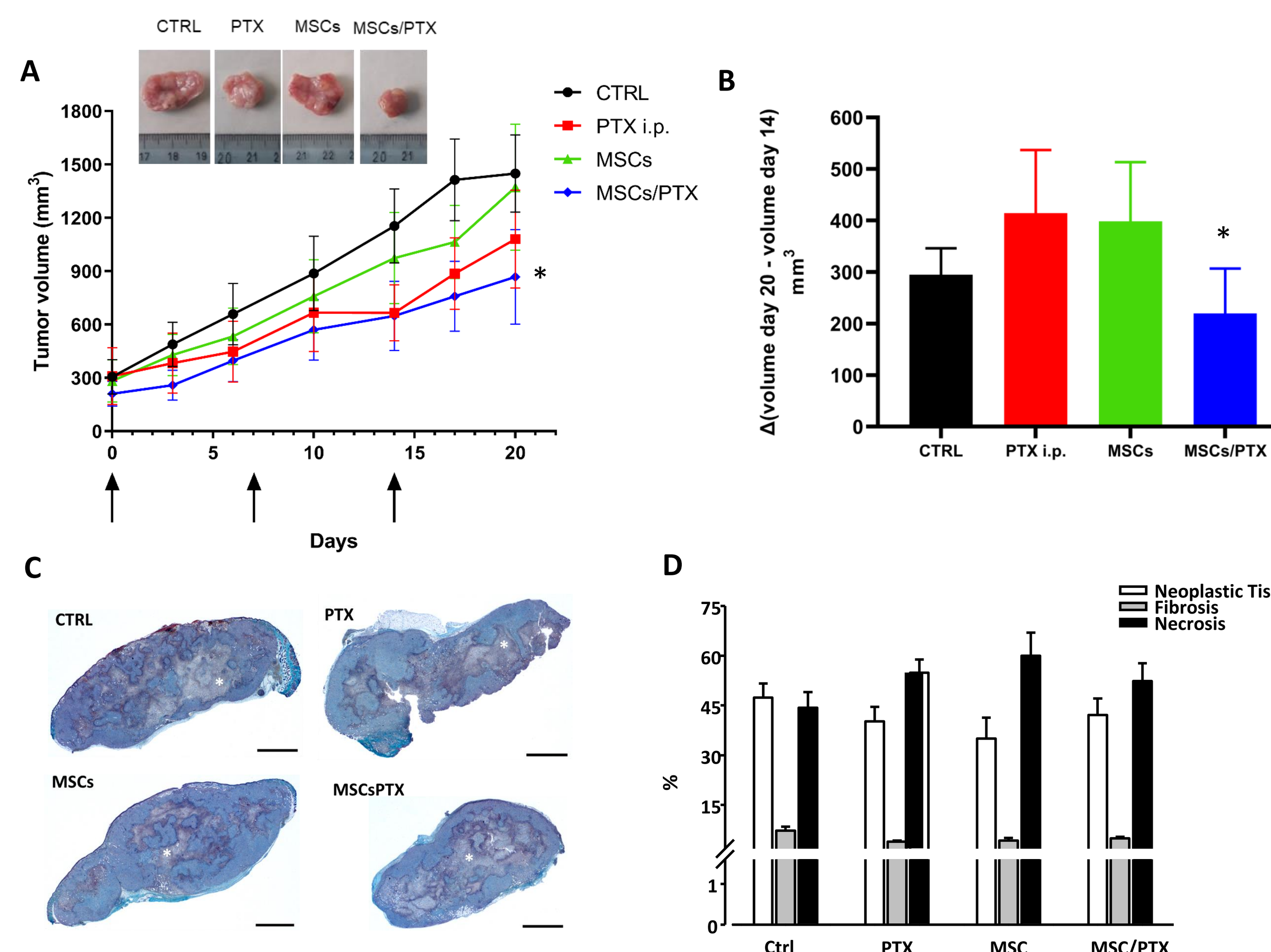

Figure 4. A. MSTO-211H cells were subcutaneously inoculated into BALB/C nude mice (tumors average size ~100 mm³) and animals were treated once a week (days 0, 7 and 14; black arrows) with vehicle alone (CTRL), PTX (10 mg/kg), 10^6 MSCs, and 10^6 MSCs/PTX; tumor volumes were measured twice per week and data are expressed as means ± SEM. Representative images of dissected xenograft tumors are reported. **B.** Values of increasing tumor volume (ΔV = volume at day 20 – volume at day 14) after stop of treatments. **C.** Morphometric analysis of tumor xenografts of Masson's Trichrome stained sections of tumor nodules. Scale bars = 2 mm. **D.** Bar graphs showing the quantitative evaluation of tissue composition (neoplastic tissue, fibrosis and necrosis). (*p<0.05).

The effect of MSCs primed with PTX was evaluated *in vivo* on mesothelioma xenografts in mice, measuring volumes of growing tumor masses. Figure 4A 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 was observed in mice treated with unprimed MSCs. After the last treatment (day 14) tumor volumes were monitored for further 6 days, and tumor progression in mice treated with MSCs/PTX was significantly (p < 0.05) lower than that observed in mice treated with PTX or MSCs (Fig. 4B). 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. 4C,D).

Conclusions

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. Our data strongly suggest that drug delivery by MSCs can be a very useful approach in the treatment of many solid tumors. 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.

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