L-Carnosine Prevents the Pro-cancerogenic Activity of Senescent Peritoneal Mesothelium Towards Ovarian Cancer Cells

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Abstract. *Background/Aim: L-Carnosine inhibits senescence of somatic cells and displays anticancer activity. Here we analyzed if L-carnosine (20 mM) retards senescence of human peritoneal mesothelial cells (HPMCs) and inhibits progression of ovarian cancer cells. Materials and Methods: Experiments were performed with primary HPMCs established from patients undergoing abdominal surgery and with three ovarian cancer cell lines: A2780, OVCAR-3 and SKOV-3. Results: L-Carnosine retards senescence of HPMCs plausibly via inhibition of mitochondria-related oxidative stress. Prolonged exposure of HPMCs to L-carnosine prevented senescent HPMC-dependent exacerbation of cancer cell adhesion, migration, invasion and proliferation, which may be linked with decreased secretion of various pro-cancerogenic agents by HPMCs. Cancer cells exposed directly to L-carnosine displayed reduced viability, increased frequency of apoptosis and unaltered proliferation. Conclusion: L-carnosine may be a valuable anticancer drug, especially in the context of prevention and therapy of intraperitoneal ovarian cancer metastasis.*

The peritoneal cavity is a prime site of ovarian cancer metastasis. In advanced stages of disease, cancer cells disseminate by ovarian surface shedding and are carried and transported by peritoneal fluid to finally sediment on the surfaces of the peritoneum (1). There is agreement that the primary role during colonization of the peritoneal cavity by ovarian cancer cells is played by interactions between the cancer cells and the normal human peritoneal mesothelium (HPMCs) (2). These interactions include cancer cell attachment to HPMCs (adhesion), dissemination over a larger area (migration), penetration across HPMCs towards tissue stroma (invasion), and multiplication in a new localization (proliferation).

It was recently found that the aggressiveness of ovarian cancer is intensified, both *in vitro* and *in vivo*, when HPMCs become senescent (3). In fact, senescent HPMCs, that accumulate with age (4), display certain features that promote various aspects of cancer cell progression. For instance, they generate increased amounts of fibronectin which improves their adhesion (5), as well as hypersecreting numerous mediators of angiogenesis, inflammation and extracellular matrix (ECM) remodelling (6, 7). Importantly, the vast majority of the above-listed features of senescent HPMCs, as well as the senescence of these cells itself, is determined by oxidative stress (7, 8).

Taking this into account, we designed a project aimed at establishing if the dipeptide, L-carnosine (β-alanyl-L-histidine), known as promising anti-senescence and anti-neoplastic agent (9, 10), retards senescence of HPMCs and prevents their pro-cancerogenic activity towards three representative lines of ovarian cancer cells (A2780, OVCAR-3, SKOV-3).

Materials and Methods

Materials. Unless otherwise stated, all chemicals (including L-carnosine) and culture plastics were purchased from Sigma (St. Louis, MO, USA).

Culture and senescence of mesothelial cells. HPMCs were isolated from pieces of omentum obtained from 12 patients undergoing abdominal surgery (Institutional Consent number 465/09), as described elsewhere (11). The reasons for the surgery included aortic aneurysm (5), hernia (4), and bowel obstruction (3). The cells were grown in medium M199 with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.4 μg/ml), and 10% foetal bovine serum (FBS).

Senescence of HPMCs was induced by serial passaging until the exhaustion of cell replicative capacity (12). At each passage, cells were counted to calculate the cumulative number of population

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doublings (CPD). HPMCs were cultured into senescence in standard medium and in a medium containing L-carnosine at the concentration of 20 mM (9,13). The media were exchanged every 3 days.

Cancer cells. The ovarian cancer cells A2780 and SKOV-3 were obtained from the European Collection of Cell Cultures (Porton Down, Wiltshire, UK) and propagated in RPMI-1640 medium with L-glutamine (2 mmol/l), penicillin (100 U/ml), streptomycin (100 g/ml), and 10% FBS. The OVCAR-3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and grown in RPMI-1640 medium with L-glutamine (2 mmol/l), HEPES (10 mmol/l), sodium pyruvate (1 mmol/l), glucose (4500 mg/l), insulin (0,01 mg/ml), and 20% FBS. Cancer cells were exposed to L-carnosine at concentrations 0-500 mM for 24 h to examine their viability and at concentrations of 20, 50 and 100 mM for 24 h to assess their apoptosis. The cells maintained in the standard growth medium without L-carnosine were used as the controls.

Detection of senescence markers in HPMCs. Senescence-associated β-galactosidase (SA-β-Gal) was detected according to Dimri *et al*. (14). The presence of histone γ-H2A.X foci was analyzed using immunofluorescence, essentially as described in (7).

Flow cytometry for oxidative stress-related parameters. Cells were harvested using trypsin-EDTA solution, washed with phosphate-buffered saline and centrifuged. The pellets were resuspended in a fresh medium and probed with fluorescent dyes (all obtained from Molecular Probes, Eugene OR, USA). To measure superoxides, cells were stained with 5 μM MitoSOX for 10 min, and the fluorescence was monitored in FL3. Cellular peroxides were detected by staining with 30 μM dihydrorhodamine 123 (DHR) for 30 min at 37˚C, and analysed in FL1. Mitochondrial membrane potential (MMP) was measured as the FL3/FL1 ratio in cells stained with 1 μg/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for 30 min at 37˚C. Mitochondrial mass was measured as FL1 fluorescence after cell staining with 10 μM of 10-*n*-nonyl-acridine orange (NAO) for 10 min at 37˚C in the dark. The measurements were made using a PAS-PPCS flow cytometer (Partec GmbH, Münster, Germany).

Collection of conditioned media and mesothelial cell secretome analysis. Conditioned media (CM) were harvested from young and senescent HPMCs. In brief, 4×10^5 of the cells were seeded into 25 cm^3 flasks, allowed to attach for 4 hours and incubated in a serum-free medium for 72 h. Samples of the CM were centrifuged, filtered through a 0.2 μm pore size filter, and stored at −80˚C until required.

Concentrations of growth-related oncogene 1(GRO1), interleukin 6 (IL6), interleukin 8 (IL8), monocyte chemoattractant protein 1 (MCP1), plasminogen activator inhibitor 1 (PAI1), transforming growth factor beta 1 (TGFβ1), and urokinase plasminogen activator (uPA) in samples of CM were measured using appropriate DuoSet® Immunoassay Development kits (R&D Systems, Abingdon, UK). The concentration of fibronectin was estimated using Fibronectin Human ELISA Kit, purchased from Abcam (Cambridge, UK).

Determination of cancer cell adhesion, migration, invasion, and proliferation. Adhesion of calcein AM-probed ovarian cancer cells to HPMCs was quantified according to a method described elsewhere (5). Cancer cell migration towards a chemotactic gradient generated by CM from HPMCs was examined with Transwell inserts (Costar, Inc., Corning, NY, USA), as described in (15). Cancer cell invasion across monolayered HPMCs lying on Matrigel was analyzed using the BD BioCoat™ Tumor Invasion Chamber (BD Biosciences, Bedford, MA, USA), as per manufacturer's instructions. Proliferation of cancer cells exposed for 24 h to HPMC-derived CM was examined using a radioisotope method, as described elsewhere (12).

Determination of cancer cell viability and apoptosis. Cancer cell viability was assessed with the 3-(4,5-dimethylthiazol-2-YI)-2,5-diphenyltetrazolium bromide (MTT) test on high-density cultures, whereas the incidence of apoptosis was quantified according to the percentage of cells in the subG1 fraction using flow cytometry [both as described in (12)].

Statistics. Statistical analysis was performed using GraphPad Prism™ v.5.00 software (GraphPad Software, San Diego, CA, USA). The means were compared with repeated measures analysis of variance (ANOVA) with the Newman–Keuls test *post-hoc*. When appropriate, the two-way ANOVA and Wilcoxon matched-pairs test were used. Results are expressed as means±SEM. Differences with a *p*-value of less than 0.05 were considered to be statistically significant.

Results

L-Carnosine delays replicative senescence of HPMCs. Long-term growth of HPMCs in standard conditions was compared with the lifespan of cells continuously propagated under 20 mM L-carnosine. Experiments showed that control cells entered senescence considerably earlier than their counterparts subjected to L-carnosine. The CPD achieved by these cells was 41±3% lower than those exposed to L-carnosine (Figure 1A). Anti-senescent effect of L-carnosine was accompanied by a significant inhibition of the development of two markers of senescence, that is SA-β-Gal and phosphorylated variant of histone H2A.X (γ-H2A.X). The fraction of pre-senescent HPMCs subjected to L-carnosine expressing SA-β-Gal and γ-H2A.X foci was lower by 60±5% ($p<0.01$) and 42±3% ($p<0.03$), respectively, compared with the control cells (Figure 1B).

L-Carnosine suppresses mitochondria-related oxidative stress in HPMCs. In order to evaluate the effect of L-carnosine on oxidative stress accompanying senescence of HPMCs, four parameters related to this phenomenon, namely mitochondrial membrane potential (JC-1), mitochondria biogenesis (NAO), production of mitochondrial superoxides (MitoSOX), and production of cellular peroxides (DHR) were studied. It was found that senescence of HPMCs was associated with decreased mitochondrial membrane potential and increased mitochondria biogenesis. In pre-senescent cells exposed to L-carnosine, mitochondrial membrane potential did not decline; on the contrary it went up to values even higher than in young cells. At the same time, it was higher by 148±8% ($p<0.001$) compared to senescent control

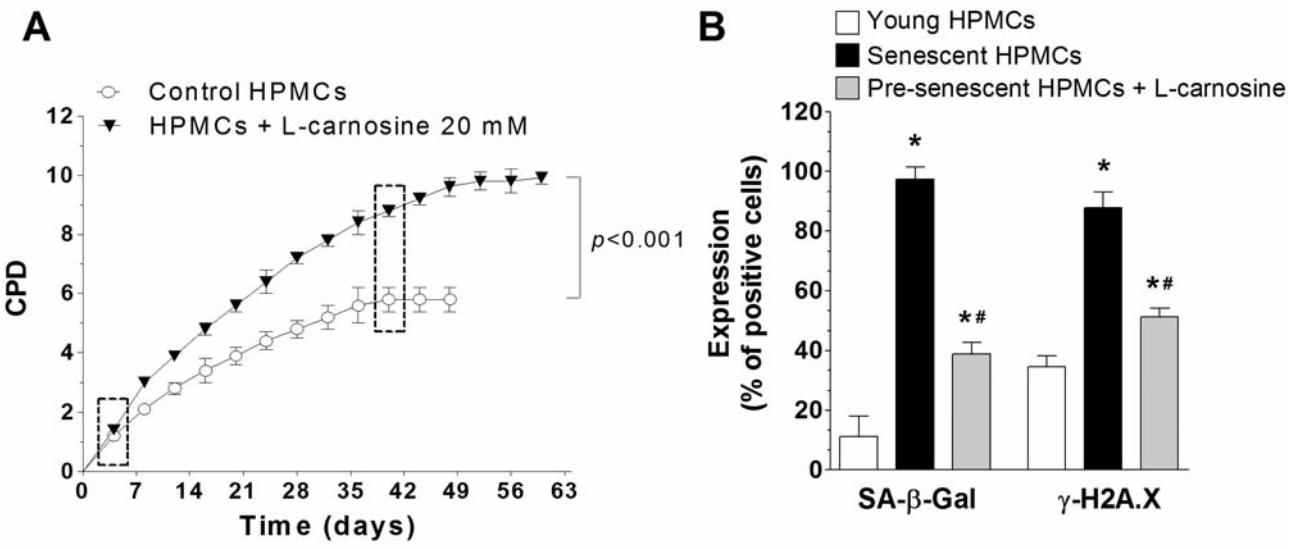

Figure 1. *Effect of L-carnosine on replicative lifespan (A) and the expression of markers of senescence (B) in cultured human peritoneal mesothelial cells (HPMCs). The boxes in panel A indicate timepoints corresponding to young cells, senescent cells, and pre-senescent cells treated with L-carnosine. The experiments were performed with HPMCs from 12 different donors. Significant differences at p<0.05 as compared with *young HPMCs and with #senescent HPMCs not treated with L-carnosine.*

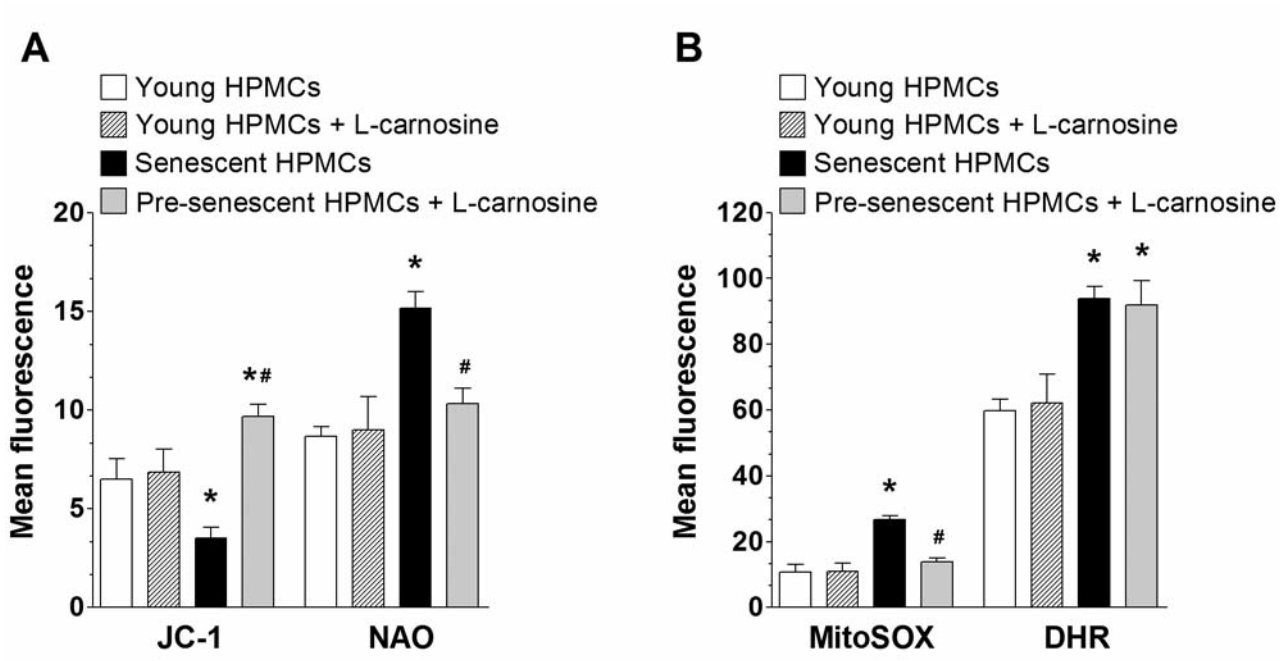

Figure 2. *Effect of L-carnosine on oxidative stress-related parameters during senescence of human peritoneal mesothelial cells (HPMCs). Significant differences at p<0.05 compared to *young HPMCs and with #senescent HPMCs not treated with L-carnosine. The experiments were performed with HPMCs from 12 different donors.*

HPMCs. When it comes to mitochondria biogenesis, it did not change during senescence of cells propagated under L-carnosine. If these cells are compared with their senescent control counterparts, the biogenesis of mitochondria in the former was lower by 33±3% (p<0.04) (Figure 2A).

Regarding the production of reactive oxygen species, the level of superoxides in pre-senescent cells subjected to L-carnosine was lower by 48±6% (p<0.003) than in cells that entered senescence under standard conditions. At the same time, it was comparable to the value characterizing young

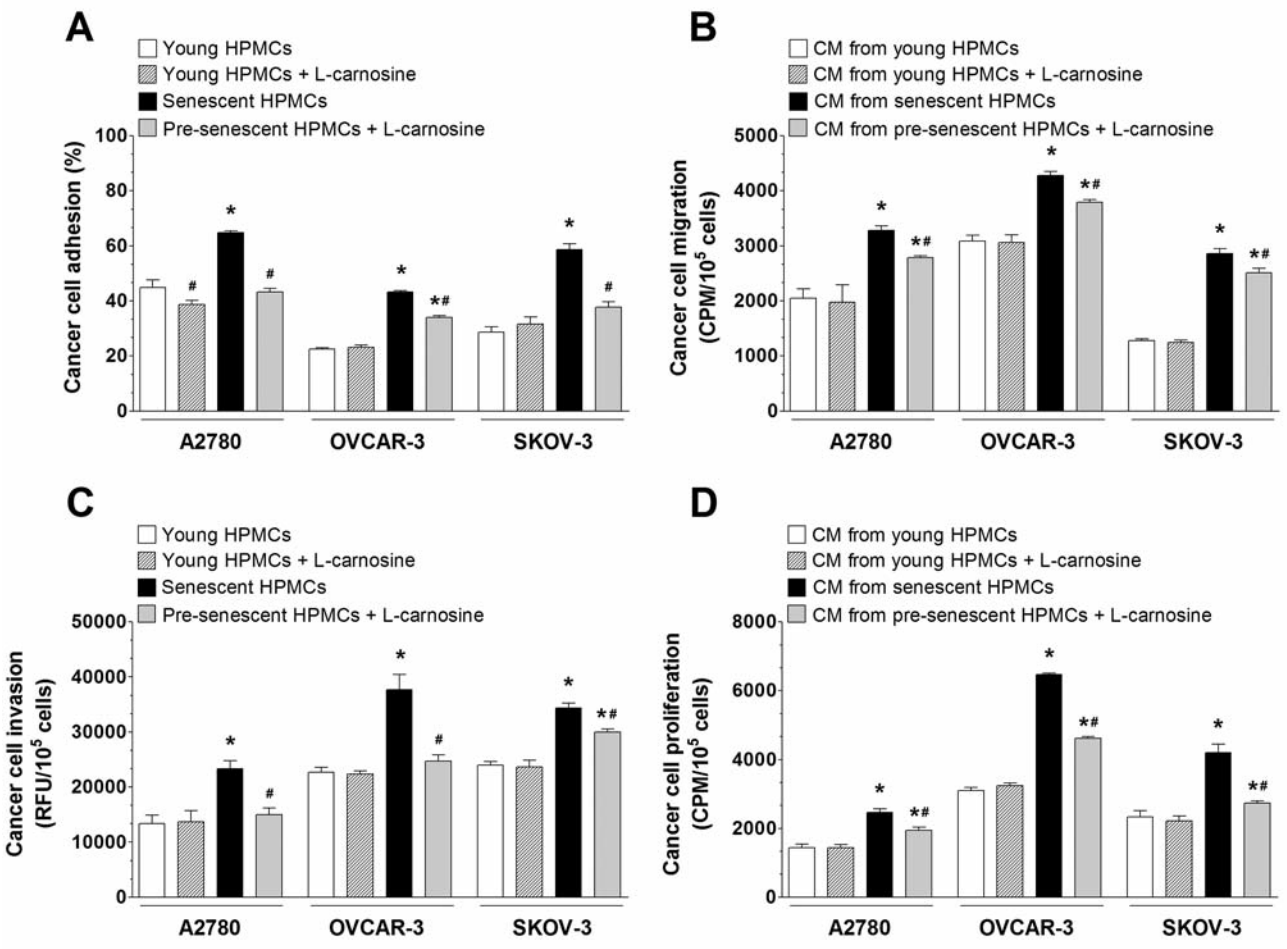

Figure 3. *Effect of L-carnosine on HPMC-dependent adhesion (A), migration (B), invasion (C), and proliferation (D) of ovarian cancer cells. Significant differences at p<0.05 compared to *young HPMCs and with #senescent HPMCs not treated with L-carnosine. The experiments were performed using HPMCs obtained from 12 different donors. Cancer cells were used in quadruplicates. RFU: Relative fluorescence units; CPM: counts per minute.*

cells. The level of peroxides increased during senescence of HPMCs, albeit the vales obtained for the control cells and those exposed to L-carnosine were similar, irrespective of the replicative age of the culture (Figure 2B).

L-Carnosine inhibits senescent HPMC-related progression of ovarian cancer cells. Cancer cell adhesion, migration, invasion and proliferation were examined in response to HPMCs senesced under standard conditions and in the presence of L-carnosine. All indicators of cancer cell progression were more pronounced when the cancer cells were exposed to CM derived from senescent HPMCs (migration/proliferation) or when they were in direct contact with senescent cells (adhesion/invasion). L-Carnosine reduced adhesion of ovarian cancer cells to pre-senescent HPMCs. In A2780 and SKOV-3 cells it prevented the senescence-associated increase in the cancer cell binding to HPMCs (Figure 3A). Migration of cancer cells towards a chemotactic gradient generated by CM from L-carnosine treated HPMCs was lower compared with values obtained for senescent control cells (Figure 3B).

The ability of cancer cells to invade Matrigel and pre-senescent HPMCs modified by L-carnosine was reduced. In A2780 and OVCAR-3 cells, L-carnosine prevented senescence-dependent increase in the invasive potential of cancer cells (Figure 3C). CM generated by pre-senescent HPMCs treated with L-carnosine stimulated proliferation to a markedly lesser extent than CM harvested from cells that senesced under standard culture conditions (Figure 3D).

L-Carnosine reduces production of pro-cancerous agents by pre-senescent HPMCs. The concentration of eight arbitrarily selected molecules, known to be involved in ovarian cancer cell progression, in CM generated by senescent HPMCs and

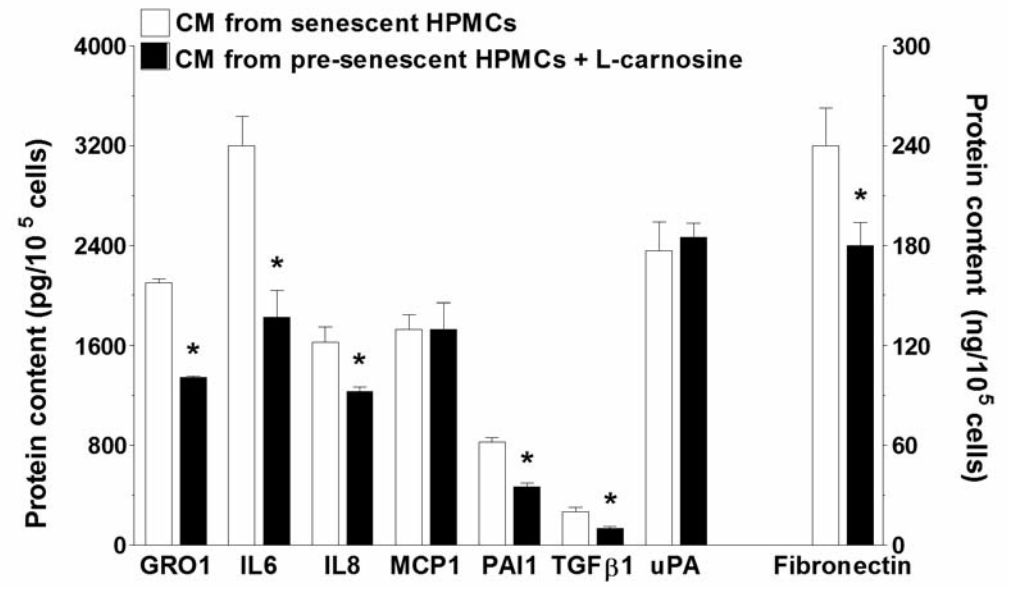

Figure 4. *Effect of L-carnosine on secretory phenotype of senescent human peritoneal mesothelial cells (HPMCs). *Significant differences at p<0.05 compared to senescent HPMCs not treated with L-carnosine. The experiments were performed using primary cultures of HPMCs obtained from 12 different donors.*

pre-senescent HPMCs subjected to L-carnosine was compared. Pre-senescent HPMCs growing under L-carnosine secreted lower amounts of GRO1 (by 36±3%, $p<0.04$), IL6 (by 43±7%, $p<0.03$), IL8 (by 24±3%, $p<0.05$), PAI1 (by 44±4%, $p<0.02$), TGFβ1 (by 50±9%, $p<0.02$), and fibronectin (by 26±3%, $p<0.05$). At the same time, L-carnosine did not affect the secretion of MCP1 and uPA (Figure 4).

L-Carnosine affects viability and apoptosis of ovarian cancer cells. In order to examine the effect of L-carnosine on viability of ovarian cancer cells, sub-confluent cultures of A2780, OVCAR-3 and SKOV-3 cells were subjected to a wide range of its concentrations (0-500 mM) for 24 h and then MTT assay was performed. L-carnosine was found to affect viability of all cancer cell lines in a cell type-specific manner. In A2780 and OVCAR-3, the lowest concentration of L-carnosine which reduced cell viability was 50 mM ($p<0.05$ for both). In the case of SKOV-3 cells, L-carnosine reduced viability starting from 100 mM ($p<0.05$) (Figure 5A). Results of cytotoxicity assay re-calculated using CalcuSyn software (Biosoft, Cambridge, UK), revealed that the half-maximal inhibitory concentration (IC_{50}) of L-carnosine, estimated was 165 mM for A2780 cells, 125 mM for OVCAR-3 cells, and 485 mM for SKOV-3 cells. At the same time, L-carnosine used at 20, 50 and 100 mM for 24 h appeared to increase the percentage of apoptotic cells. In A2780 and OVCAR-3 cells this happened at 50 mM ($p<0.04$ and $p<0.05$, respectively) and 100 mM ($p<0.03$ and $p<0.04$, respectively), whereas in SKOV-3 cells it occurred at 100 mM ($p<0.05$) (Figure 5B). The proliferative capacity of ovarian cancer cells exposed to L-carnosine remained unchanged (Figure 5C).

Discussion

L-Carnosine is a naturally-occurring dipeptide, widely distributed in human tissues. Although it is small molecule, it has various biological properties, including antioxidative and anti-glycation activity, chelation of metals, regulation of pH, and protection of DNA (16). Moreover L-carnosine extends the replicative lifespan of somatic cells (9), as well as restricting the growth of cancer cells both *in vitro* (10) and *in vivo* (17). Interestingly, though the anti-senescence and anticancer properties of L-carnosine have already been well documented, its effect on senescence-related cancer cell progression has never been studied before.

In this report, we showed that 20 mM L-carnosine restricted behaviour of three representative lines of ovarian cancer cells (A2780, OVCAR-3, SKOV-3), initially aggravated by senescent HPMCs. This effect is primarily based on its ability to extend the replicative lifespan of HPMCs and, similarly as in fibroblasts (9), to reverse their senescence phenotype. Senescence of HPMCs differs markedly, however, from that of fibroblasts, as in the former it proceeds in a telomere-independent fashion (18). For this reason, the mode by which L-carnosine delays senescence of HPMCs is likely not related to the protection of telomeric

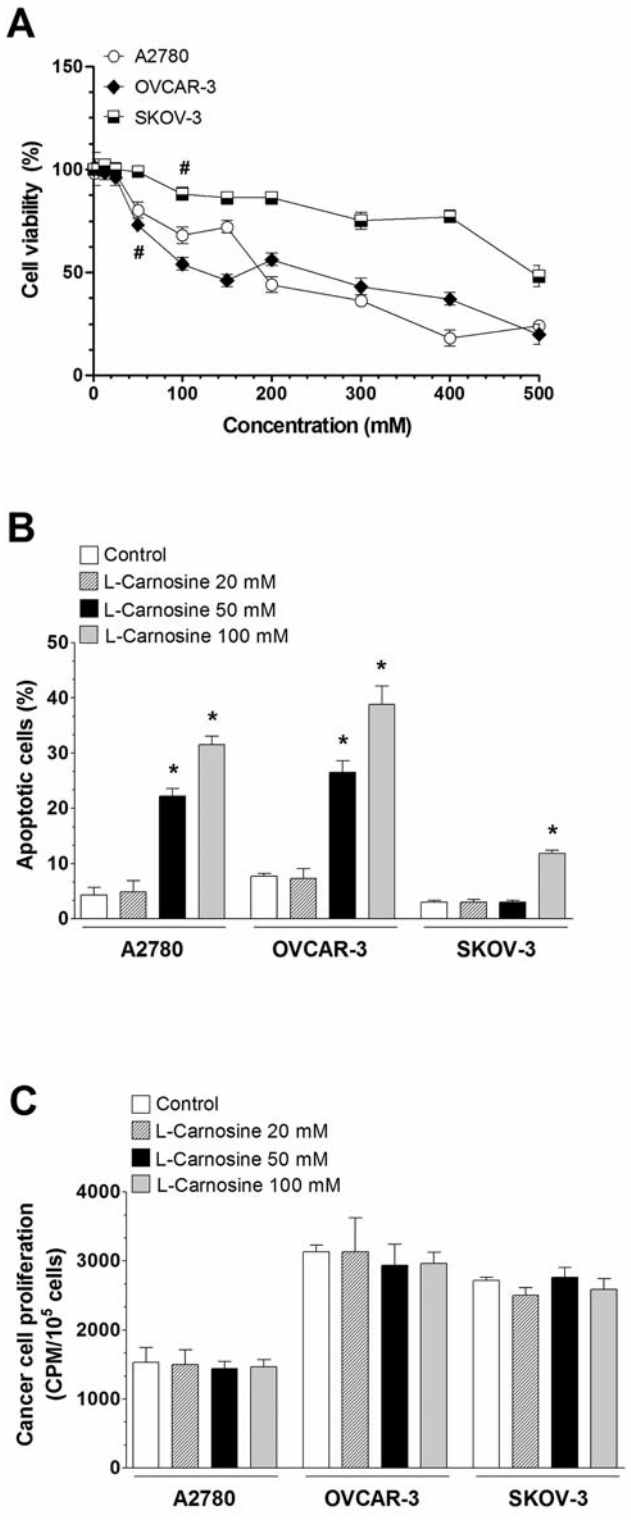

Figure 5. *Effect of L-carnosine on viability (A), apoptosis (B) and proliferation (C) of ovarian cancer cells. Significant differences at p<0.05 compared to *control cells not treated with L-carnosine. #Concentration of L-carnosine reducing cancer cell viability. The experiments were performed with eight separate cultures of ovarian cancer cells. CPM: Counts per minute.*

DNA (13). On the other hand, HPMCs are very prone to oxidative stress, which controls their senescence (19). Interestingly, L-carnosine appeared to reduce mitochondrial oxidative stress, as it improved cell ability to generate ATP (increased membrane potential), which elicited a compensatory decrease in mitochondria biogenesis and in the production of superoxides. These findings are intriguing because L-carnosine is known to inhibit glycolysis and generation of ATP in cancer cells (20). Taking into account, however, profound differences in the activity of many important metabolic enzymes in cancer and normal cells (21), this opposing action of L-carnosine on the ATP pool in ovarian cancer cells and HPMCs seems logical.

Returning to senescent HPMC-dependent cancer cell progression, L-carnosine inhibited its four major elements, namely adhesion, migration, invasion and proliferation. This activity of L-carnosine was associated with its ability to modify the secretory phenotype of senescent HPMCs (7). In fact, somatic cells senesced under standard conditions secrete increased amounts of multiple agents (cytokines, chemokines, growth factors, ECM-remodeling molecules) that create a specific, inflammation-like environment in which vital elements of cancer progression are intensified (22). HPMCs whose senescence was delayed by L-carnosine displayed down-regulated secretion of proteins known to mediate the interactions between HPMCs and ovarian cancer cells. These included fibronectin responsible for ovarian cancer cell adhesion (5); IL6, IL8 and GRO1 known to stimulate cancer cell proliferation and migration (23, 24); and PAI1 and TGFβ1 involved in cancer cell invasion (25, 26).

In the last part of the project, L-carnosine was directly applied to ovarian cancer cells and then their viability, proliferation and apoptosis were quantified. The study showed that L-carnosine exerts cytotoxic activity towards all three ovarian cancer cell lines studied. This effect was plausibly associated, at least to some extent, with an increased frequency of apoptosis. Regarding cancer cell proliferation, L-carnosine failed to affect this process. Interestingly, in gastric cancer cells, L-carnosine reduced proliferation and failed to modify viability and apoptosis (27), which may suggest that effects of this compound against cancer cells are cell-specific.

Our report provides evidence that L-carnosine has the ability to restrict the behaviour of ovarian cancer cells, related to the pro-cancerous phenotype of senescent HPMCs. This is, in turn, associated with its ability to delay the onset of HPMC senescence, plausibly via the normalization of mitochondrial metabolism and reduction of mitochondrial oxidative stress. Validation of these findings using animals is now of special importance to confirm these findings *in vivo*, and such experiments are currently in progress.

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