EXPERIMENTAL STUDY

Inhibitory effects of mouse bone marrow mesenchymal stem cell soup on staurospurine-induced cell death in MCF-7 and AGS

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ABSTRACT

BACKGROUND: Staurospurine induces apoptosis in cell line. Bone Marrow Mesenchymal stem cells Soup is a promising tool for cell proliferation via a variety of secreted factors. In this study, we examined the effects of BMSCs Soup on Staurospurine induced-cell death in MCF-7 and AGS cells.

METHODS: There were three Groups: Group I: no incubation with BM Soup; Group II: incubated with 24 h BM Soup; Group III: incubation with 48 h BM Soup. There were two treatments in each group. The treatments were 1μM Staurospurine (Treatment 1) and 0.0 μM Staurospurine (Treatment 2). The cells were cultured in culture medium containing 0.2 % BSA. We obtained the cell viability, cell death and NO concentration.

RESULTS: Our results showed that BM soup administration for 48 hours protected against 1μM staurosporine concentration induced cell death and reduced cell toxicity in MCF-7 and AGS cells. Cell viability and cell toxicity assay showed that BM soup in time dependent manner increased cell viability (p < 0.05) and cell death assay showed that cell death in time dependent manner was decreased(p < 0.05). Our data showed that BM soup with increasing NO concentration reduced staurospurine induced cell death and cell cytotoxicity (p < 0.05).

CONCLUSION: It’s concluded that BMSCs soup suppressed staurospurine-induced cytotoxicity activity process in MCF-7 and AGS cells (Fig. 9, Ref. 79). Text in PDF www.elis.sk.

KEY WORDS: BM-MSC soup, cell viability, cell death, NO.

Introduction

Many reports proposed that adult bone marrow mesenchymal stem cells (BM-MSCs) can differentiate into osteoblasts, chondroblasts, adipocytes, and hematopoiesis supporting stroma (1–3). Besides the mesenchymal tissue, many studies demonstrated that MSCs could differentiate into various non-mesenchymal tissue lineages under appropriate experimental conditions in vitro and in vivo, such as: hepatocytes (4, 5), cardiomyocytes (6, 7), lung alveolar epithelium (8), even neuron and glia (9–13). Recently, the amazing potential of MSCs attracted intense interest in the possible applications of MSCs in cell and gene therapy for disease, because mesenchymal Stem Cells can be obtained from bone marrow easily and expanded rapidly in vitro (14, 15). It has been shown that MSCs could easily induce differentiation in vitro by chemical inducers (9), cytokines (4, 16, 17), co-culture with neurons (18, 19), chemical inducers plus cytokines (10, 20) and transfect plus cytokines (21), etc.

Many studies had reported that MSCs transplantation improved cellular and functional recovery, increased endogenous cell proliferation, decreased apoptosis, promoted reduced lesion size and angiogenesis of neuronal cells (22) in CNS injuries, such as stroke and spinal cord injury in animal models. It showed that transplanted MSCs might exert beneficial effects in injury including their ability to produce growth factors (22–24). However, MSCs can secrete a variety of molecules including trophic factors and antiapoptotic molecules, which may provide the main mechanism responsible for their therapeutic effect (25). In addition, MSCs can inhibit the release of proinflammatory cytokines and promote the survival of damaged cells (26). For example, the therapeutic benefit of MSC cytokines has been observed in acute lung injury (27, 28), myocardial infarction (29), acute renal failure (30), cerebral ischemia (31, 32) and Alzheimer’s disease (33).

On the other hand, the most recent mechanism of action is that BMSC cells provide a local paracrine effect (34–36) such as tissue regeneration and improvements. This have been proposed as paracrine effect of stem cell action (35–37). However, after closer investigation, we could not fully explain organ improvement.

Meanwhile, several studies have shown that stem cells soup include soluble factors that act a paracrine fashion, contribute to organ repair and regeneration. These factors are cytokines; growth and other factors inducing cytoprotection, neovascularization, and medium endogenous tissue regeneration via activation of resident tissue stem cells. In addition, tissue re-modelling and organ function is affected by these paracrine factors (35).

Recent study showed that BMSC soup paracrine mechanism is the main cause behind the reported improvement of salivary organ

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function (38). Many studies showed that once MSCs have promoted the development of fibroblasts, endothelial cells and tissue progenitor cells, which carried out tissue regeneration and repair by secretion of paracrine factors, including cytokines such as TNF-a, IL-1, IFN-g, toxins of infectious agents and hypoxia can stimulate the release of many growth factors by MSCs, including EGF, FGF, PDGF, TGF-b, VEGF, HGF, IFG-1, keratinocyte growth factor (KGF) and stromal cell derived factor-1 (SDF-1) (39–42).

There has been no evidence about any effect of mBMSC Soup on improving any form of the staurosporine induced apoptosis or cell death in breast and gastric cell line. The aim of this experimental study was to investigate the efficacy of mBMSC Soup on this proposal. In this context, we aimed to test the efficiency of mBMSC Soup in suppressing the staurosporine induced cell death in MCF-7 and AGS cell lines. The hypothesis is that mBMSC Soup protects cells and increases cell viability.

Material and methods

Preparation of mouse bone marrow cells

Isolation and culture of mBMSCs were carried out as previously described (43). Bone marrow was obtained from 6–8-week-old NMRI mice. For isolation of MSCs, tibias and femurs were dissected and the ends of the bones were cut. The marrow was expressed with 0.2 % BSA. The cell pellet was re-suspended in DMEM culture medium, washed, and stained with methylene blue for 2 hours to identify all colonies. To evaluate the in vitro differentiation ability of cultured mBMSCs, cells were trypsinized, harvested, and plated at 5 × 10^4 cells per well in 24-well plates in standard medium. Medium was replaced 24 hours after plating. Osteogenic differentiation was induced in mBMSC cultures by adding ascorbic acid (50 μg/ml), sodium b-glycerophosphate (10 mM), and dexamethasone (10^-4 M) in standard medium. After 2 weeks, plates were washed with PBS, fixed with 4 % paraformaldehyde, and stained with 1 % alizarin red. Adipogenic differentiation was induced in another aliquot of cells of the initial culture by the addition of dexamethasone (10^-3 M) and insulin (6 ng/ml), in DMEM medium supplemented with 1 % foetal calf serum, 1 % glutamine, and 1 % penicillin-streptomycin to the cell culture.

After 3 weeks, plates were washed with PBS, fixed with 4 % paraformaldehyde.

For von Kossa staining, cells were incubated in 5 % silver nitrate in the dark for 30 minutes, and then they were exposed to ultraviolet light for 1 h. The secretion of calcified ECM was observed as black nodules with von Kossa staining.

mBM soup preparation

After confirming and harvesting the mBMSC cells, mBMSC condition medium as mBM Soup was prepared. Briefly, mBMSCs cells were cultured in DMEM culture medium, culture medium was replaced every 2 or 3 days about 2 weeks. When cell reached to 80 % confluence, the medium was changed by fresh DMEM free serum and 0.2 % BSA culture medium. The cells were maintained for 24 h or 48 h. Then, the condition mediums were harvested as mBM soup (mBM soup 24 and mBM soup 48 h, respectively). Protein concentration of mBM Soup was 0.41 μg/ul (from 10^7 cells). mBM Soup was kept on –70 °C until further uses.

Cell line

MCF-7 and AGS cells were grown in a 25-cm 2 tissue culture flask in DMEM culture medium (Gibco), supplemented with 10 % foetal bovine serum (FBS, Gibco; UK), 1 % NEAA (Sigma), 100 μ/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma). The cells were maintained at 37 °C in a humidified, 5 % CO₂ environment.

Cell treatment

One day after plating the MCF-7 and AGS cells, the cells were washed with PBS, pH 7.4. There were six treatments. Treatment 1: 1 μM Staurosporine, Treatment 2: control cells, Treatment 3: mBM Soup 24 h, Treatment 4: mBM Soup 24 h together with 1 μM Staurosporine, Treatment 5: mBM Soup 48 h and Treatment 6: mBM Soup 48 h together with 1 μM Staurosporine.

Then, the cells were placed in the incubator at 37 °C with 5 % CO₂. The cells were cultured in DMEM culture medium containing 0.2 % BSA.

Cell viability measurement

Trypan blue is a vital stain that leaves nonviable cells with a distinctive blue colour when observed under a microscope, while
viable cells appear unstained. Viable cells have intact cell membranes and hence do not take in dye from the surrounding medium. On the other hand, nonviable cells do not have an intact and functional membrane and hence do take up dye from their surroundings. This results in the ability to easily distinguish between viable and nonviable cells, since the former are unstained, small, and round, while the latter are stained and swollen. The method does not differentiate between apoptotic and necrotic cells.

The traditional method of performing trypan blue (0.4 g/100 ml in PBS) cell viability analysis involves manual staining and use of a hemocytometer for counting (44).

**MTT assay**

To perform the test, 15×10⁵ MCF-7 and AGS cells were loaded into a 96-well plate and 200 μL of DMEM medium containing 0.2 % BSA was added. After 24-hour incubation, 200 μL of treatments medium as described was added to the wells. The cells were separately incubated with different treatments medium for 24 and 48 hours.

After incubation, the MTT test was performed in which the supernatant from each well was removed and 50 μL of MTT solution (5 mg/mL) was added to each well and incubated for 3 hours. The supernatant from each well was then removed and 100 μL of dimethyl sulfoxide was added to dissolve the formazan crystals at room temperature for 30 minutes. The optical density of each well was measured using a microplate reader (EL800; USA) at 570 and 630 nm.

The viability of the cells for each concentration was calculated using the following formula (45):

\[ \text{Cell viability} \% = \left( \frac{A_{570,630, \text{sample}}}{A_{\text{control}}} \right) \times 100 \]

**NO assay**

NO was measured using the Griess staining method. For each sample, 400 μL of thawed supernatant was deproteinized by adding 6 mg of zinc sulfate. The samples were centrifuged at 4 °C and 12,000 g for 12 minutes. Separately, 100 μL of 0, 6.25, 12.5, 25, 50, 100, and 200 μM sodium nitrite was poured into standard wells. We then added 100 μL of the deproteinized samples to each well, and 100 μL of vanadium chloride, 50 μL of sulfanilamide, and 50 μL of N-(1-naphthyl) ethylenediamine dihydrochloride were added to all wells (standard and samples). All wells were incubated for 15 minutes and were assessed using a microplate reader (EL800; USA) at wavelengths of 570 and 630 nm (46).

**Quantification of cell death incidence**

Hoechst/PI nuclear staining was carried out as previously described (47). Briefly, cells were plated in 24 well cultured plates with 5×10⁴ cells/well density overnight. Then, cells were treated with different treatment mediums for a range of times in differentiation medium (6, 12, 24 and 48 h). Then cells were incubated for 30 min at 37 °C with Hoechst 33342 dye (10 ng/ml in PBS), washed twice in PBS. PI (50 ng/ml in PBS) was added just before microscopy. Cells were visualized using an inverted florescence microscope (Olympus IX-71, Japan). Nuclear morphology was scored as follows: 1, viable cells had blue stained nuclei with smooth appearance; 2, viable apoptotic cells had blue-stained nuclei with multiple bright specks of condensed chromatin; 3, non-viable apoptotic cells had red-stained nuclei with either multiple bright specks of fragmented chromatin or one or more spheres of condensed chromatin (significantly more compact than normal nuclei); 4, non-viable necrotic cells had red-stained, smooth...
and homogeneous nuclei that were about the same size as normal (control) nuclei. The cell death index was calculated by the fraction of numbers of apoptotic cells on the total cell count in 100 (300 cells), respectively.

**Data analysis**
Data are reported as the mean ± SEM. Differences among treatment groups were tested using one-way ANOVA followed by Tukey’s test. \( p < 0.05 \) was considered statistically significant.

**Results**

**Characteristics of BMSCs from NMRI mice**
The cells as mBMSCs were derived from female and male NMRI mice. The 5th passage of cells had similar morphology. Most of the mBMSCs cells were spindle-shaped similar to the fibroblast-like cells. The cells exhibited a rapid growth with cell clustering. We used Flow cytometry to detect the phenotype of the 5th passage cells. The results showed that about 98% cells of mBMSCs are CD90-positive and lack the expression of CD14, CD45 and CD34. These results showed that mouse Bone marrow cells had the characteristics of mesenchymal stem cells (Fig. 1).

Multipotency of bone marrow mesenchymal stem cells were tested and specified by differentiation assay. Differentiation assays showed that isolated mBMSC cells could be induced into osteoblast and adipocytes, as evinced by Alizarin red, Von Kossa for detection of osteogenic differentiation and Sudan III staining for adipogenic differentiation. Adipogenic differentiation in mBMSC resulted in distinct, round lipid vacuoles (Fig. 2) visualized with Sudan III. In contrast, osteogenic differentiation of mBMSC led to a considerable increase of extracellular matrix (ECM) deposits consisting of hydroxyapatite, calcium and magnesium salts stained dark red with Alizarin Red (AR) (Fig. 3). A detailed analysis of the osteogenic differentiation was accomplished by von Kossa staining.

Von Kossa staining revealed a distinct positivity for mBMSC, which was confirmed by AR-staining. Both stainings demonstrated the presence of calcite crystals as a sign of matrix ossification during osteogenic differentiation.

**Cell viability**
Comparison of the mean MCF-7 and AGS cell viabilities analysed using the trypan blue method after 6, 12, 24 and 48 hours indicated a significant difference between the treatments \( p < 0.05 \).

After 6, 12, 24 and 48 hours, the percentage of cell viability in treatments 1, 4 and 6 were decreased compared to the treatment 2 (97%) \( p < 0.05 \). After 6, 12, 24 and 48 hours the percentage of
cell viability were not different in the treatments 3 and 5 compared to the treatment 2. The percentage of cell viability was increased in the treatment 3 compared to the treatment 4 \( p < 0.001 \) (Fig. 4). The percentage of cell viability was increased in the treatments 4 and 6 compared to the treatment 1 \( p < 0.05 \).

Comparison of the mean cell viability analysed using the MTT assay after 24 and 48 hours revealed a significant increase between the treatments compared to the treatment 1, respectively \( p < 0.05 \). Cell viability was again significantly decreased by increasing the time in the treatment 1 compared to the treatment 2 \( p < 0.05 \). The percentages of cell viability were increased in the treatments 4 and 6 compared to the treatment 1 \( p < 0.05 \). The percentage of cell viability was lowest in the treatment 1 and was highest in the treatments 3 and 5 \( p < 0.05 \) (Fig. 5).

**NO levels**

NO concentration was evaluated using the Griess method. The effect of different concentrations of staurospurine on MCF-7 and AGS cells after 24 and 48 hours indicated a time-dependent decrease in NO secretion compared to the control treatment, respectively \( p < 0.05 \).

Figure 6 shows NO concentration amounts (\( \mu \text{M} \)) in the culture medium of MCF-7 and AGS cells that contained staurospurine (1 \( \mu \text{M} \)) plus different mBM soup for 24 and 48 incubation times.

After 24 and 48 h incubation, in the treatment 1, the NO concentration was decreased in the medium compared to the control sample (treatment 2), respectively \( p < 0.05 \). After the 24 and 48 h incubation, NO concentration significantly increased when cells were treated with treatments 2–6 compared to the treatment 1 \( p < 0.05 \). Data showed that the highest NO concentration was in the treatment 6 and the lowest concentration was in the treatment 1 in culture medium for 24 and 48h, respectively \( p < 0.05 \) (Fig. 6).

**Cell death indexes**

The cell death index of MCF-7 and AGS cells treated with different treatments showed an increase from the treatment 1 to the other treatments in time order, respectively \( p < 0.05 \). After 6, 12, 24 and 48h, the cell death index was highest in the treatment 1 and lowest in the treatments 3 and 5, respectively \( p < 0.05 \). The cell death index was increased in the treatment 1 compared to the treatment 2, respectively \( p < 0.05 \). The cell death index of MCF-7 and AGS cells in the treatment 4 was increased compared to the treatment 3 \( p < 0.05 \). The cell death index of cells in the treatment 6 was increased compared to the treatment 5 \( p < 0.05 \).
The cell death index of MCF-7 and AGS cells was decreased in the treatments 2, 3 and 5 compared to the treatment 1 after 6-48h incubation, respectively (p < 0.05) (Figs 7–9).

Discussion

In the present study, we showed that administered mBM Soup was capable multiple effectively: (1) to enhance the cell viability, (2) to enhance the NO concentration in culture medium, and (3) to suppress cell death or cell death index in cells lines.

Here, for the first time, we showed that administrations of mBM Soup were effective for prevention of Staurospurine-induced cell death in MCF-7 and AGS cells. Mesenchymal Stem Cells might exhibit extremely different functions in distinct tumour microenvironments because various signalising pathways can be activated in these cells. Highly controversial results have been reported in prior studies that addressed the role of MSCs in tumour development. For example, MSCs can inhibit tumour cell proliferation when co-cultured with tumour cells in vitro. Ramasamy et al has showed that MSCs could inhibit the proliferation of leukaemia cell lines and solid tumour cell lines in vitro.
(48). The results showed that MSCs could also induce apoptosis in tumour cells (49) by upregulation of caspase 3, an apoptosis-related protease (50). It has been shown that MSCs indirectly suppressed tumour growth by inhibiting angiogenesis in vascular endothelial cells (51, 52) or by directly inhibiting vascular network formation (53).

However, opposing results had been documented. For example, in PC3 cells (an animal model of prostate cancer) MSCs promoted fibroblast growth factor 2 (FGF2) secretions. It has been shown that MSCs promoting tumour angiogenesis by enhance the expression of endothelin-1 in colon cancer cell lines (54, 55). It has been shown that co-culturing gastric cancer cell lines with MSCs increased the proportion of CD133-positive gastric cancer cells (56, 57). Therefore, in this study, we tried to examine the effect of BM-MSC Soup (cell free system) on a cancer cell line (MCF7 and AGS). It is very important to examine how mBM Soup in different states affects tumour cell lines and it can also show the growth factor supporting role for the cells against the apoptosis inducers.

In the present study, the enhancing and supporting effect mBM Soup on a breast cancer cell line and gastric cell line was associated with NO increase. Yin et al has showed that the inhibitory effect on MCF7 cells was due to decreasing NO secretion (58), although decreasing cyclooxygenase 2 and cytokine expression are additional factors for consideration.

Staurosporine, a broad -spectrum protein kinase inhibitor (59), has been used to induce cell death in a wide range of cell types at 1μM concentration (60, 61). The exact mechanism responsible for staurosporine-induced cell death is not known. Several authors showed that the exact mechanism responsible for staurosporine-induced cell death in treated cells depends on calcium ions, but several controversial reports exist about the effects of staurosporine on extra or intracellular calcium sources on different treated cells. For example, it has been showed that staurosporine induces cell death by increasing cytoplasmic Ca2+ concentration, which is responsible for releasing mitochondrial cytochrome C, caspase-3 activation (62), intracellular ROS accumulation (63), and increasing (Ca2+) (64) and cell death in various cell types (eg. PC12 cells). Recently, we showed that that endogenous ROS generation, extra-cellular Ca2+, and calcium homeostasis regulation were related by mechanisms playing an important role in regulation of STS-induced cell death (65, 66).

Recently, we reported that inhibition of IP3R and PLCγ led to attenuating of the rise in intracellular calcium triggered by staurosporine, thus strongly suggesting that ROS generation could potentiate the observed intracellular calcium overload (67). Our data showed that staurosporine at 1μM concentration induced cell death and reduced cell viability by increasing the NO secretion. Maybe, that staurosporine upregulates NOS biosynthesis and intracellular calcium overload, but it needs to be investigated for these effects. This paper highlights the importance of the paracrine effects of BMSC soup on tumour cell lines, and that intact BM cells may not be necessary. Previous studies showed that paracrine cross-talking between salivary gland (SG) cells and other cell populations (such as MSC or amniotic epithelial cells) was demonstrated by using a culture system that physically separated the cell populations (68, 69). These findings on the paracrine effects of MSC showed that organ repair was due to the secretion of cytokines, chemokines, and growth factors (70). It had been showed that injecting conditioned medium from MSC cultures exerted cardiomyocyte protection and improved cardiac function in mouse infarcted hearts (71, 72). Administering mBM Soup does not require the injection of cells, which carry the risk of differentiating into unwanted/tumorigenic cell types in organs and is not patient-specific. Study showed that BMSC Soup included all cell types of the whole BM and consequently numerous proteins, cytokines and paracrine factors (73, 74).

It has been showed that TGF-β expression in MSCs promotes tumour growth of prostate cancer cell lines in mice. This tumour growth-promoting effect of MSCs can be blocked by siRNAs against TGF-β (75). Cell survival factor inhibits cell apoptosis through activating specific signalising pathway(s), including the PI3K/Akt pathway. It has been showed that transfection of constitutively active Akt prevents cell apoptosis while a dominant negative Akt induces cell apoptosis (76). In the other hand, recent studies showed that inhibitors of the PI3K/Akt pathway can sensitize cells to apoptotic stimuli (77, 78). For example, Osaki et al, showed that inhibition of PI3K caused inhibition of cell proliferation without induction of cell apoptosis and that inhibition of the PI3K-Akt signalling pathway significantly increased the sensitivity of cell apoptosis (79).

It has been showed, that BMSC Condition medium enhanced Erk1/2 and Akt phosphorylation in neurons, and the specific MEK1 inhibitor PD98059 or the phoshoinositide-3 kinase (PI3-K) inhibitor Ly294002 abolished the neuroprotective effect from staurospporine induced cell death. They showed that these findings demonstrated that BMSC trigger endogenous survival signalising pathways in neurons that mediate protection against apoptotic insults (79).

Moreover, the interaction between breast and gastric cells and mBM Soup were not clear. Our results provided evidence that mBM soup was capable to suppress cell death and induced cell viability MCF-7 and AGS cells. Studying the paracrine factors that are differentially produced in BM- MSC like NO, TGFβ and activation of PI3K/Akt, could induce the effects, but it is not really clear.

Conclusion

According to our results in the present study, application of mBMSC Soup enhance the cell viability, NO concentration and decrease cell death.

In conclusion, mBMSCs Soup suppressed the staurosporine induced cell death in breast cancer cell line and gastric cell line in a time-dependent manner. mBMSCs Soup also enhanced the proliferation of cells. It seems that the Soup exerts its effect through an induction of NO production and induction of proliferation. However, more factors need to be investigated in these effects.
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