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Mesenchymal Stem Cells Combined With IFNγ Induce Apoptosis of Breast Cancer Cells Partially Through TRAIL

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Abstract. *Background: Mesenchymal stem cells (MSCs) have gained remarkable attention because of their ability to dualistically regulate tumor growth. The main objective of this study was to evaluate the apoptotic effects of human bone marrow-derived (hBM) MSCs in combination with interferon gamma (IFN-γ) on MCF-7 breast cancer cells, and to determine the cytokines involved in the apoptotic process. Materials and Methods: hBM-MSCs were co-cultured with MCF-7 cells either directly and indirectly for 72 h in-vitro. Levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), apoptosis and cytokines were analyzed. Results: hBM-MSCs increased the apoptosis of MCF-7 cells partially through TRAIL in vitro. IFN-γ enhanced the apoptotic effect of hBM-MSCs ($p<0.001$). Conclusion: hBM-MSCs in combination with IFN-γ might be a suitable therapy for breast cancer.*

Mesenchymal stem cells (MSCs) are self-renewing cells than can differentiate into various cell types such as hepatocytes, myocytes, osteoblasts, adipocytes, chondrocytes, or neuronal cells, under specific conditions (1). Furthermore, MSCs can exhibit a marked tropism for tumors. Because of their tumor-homing properties they are also currently being tested as vehicles for delivering anticancer agents (2). Data obtained through high-throughput technologies have helped us to understand the characteristically unique nature of MSCs and it is increasingly being realized that MSCs are 'double-edged swords'. Studies have reported that MSCs can promote metastasis (2-4), impair the function of a variety of immune cells, increase fibrovascular network, support tumor vascularization (5) and enhance drug resistance (6-8).

On the other hand, increasing evidence has shown that MSCs can efficiently inhibit cancer cell proliferation and metastasis (9-12). For instance, MSCs can target gliomas with high tumor specificity and markedly enhanced survival time of tumor-bearing animals. Human MSCs home to the sites of Kaposi's sarcoma, and prevent tumor growth powerfully *in vivo* by down-regulating the activity of AKT serine/threonine kinases (AKT). Constitutively active AKT severely interfered with tumor growth-inhibitory effects of MSCs in mice xenografted with PC-3 cells (13).

Interferons (IFNs) are functional cytokines which control cellular and immune responses as well as antiviral and antitumor activity. They comprise two groups: Type I and type II IFNs. Type I IFNs (IFNα and IFNβ) potently suppress tumor cell growth and induce apoptotic cell death. Type II interferons, which includes IFNγ, have antiviral effects.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has attracted considerable attention because of its ability to selectively target cancer cells.

Rapidly emerging experimental evidence is deepening our understanding related to causes of the failure of a soluble and strictly trimeric TRAIL. It is now more understandable that full benefits of TRAIL-based therapeutics are not being reaped due to a very short serum half-life and improper receptor crosslinking (14). In accordance with this approach, dimeric epidermal growth factor receptor-specific diabody single-chain (Db-sc) TRAIL was expressed in MSCs. Single subcutaneous injection of MSCs was given to nude mice to verify the presence of Db-scTRAIL in serum fractions. TRAIL signals appeared after 7 and 14 days in the range of 1.5 ng/ml in these mice (14).

Furthermore, intramuscularly administered adherent stromal cells significantly inhibited progression of tumor and vascularization in mice xenografted with MDA-MB-231

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Key Words: MCF-7, breast cancer, mesenchymal stem cells, TRAIL.

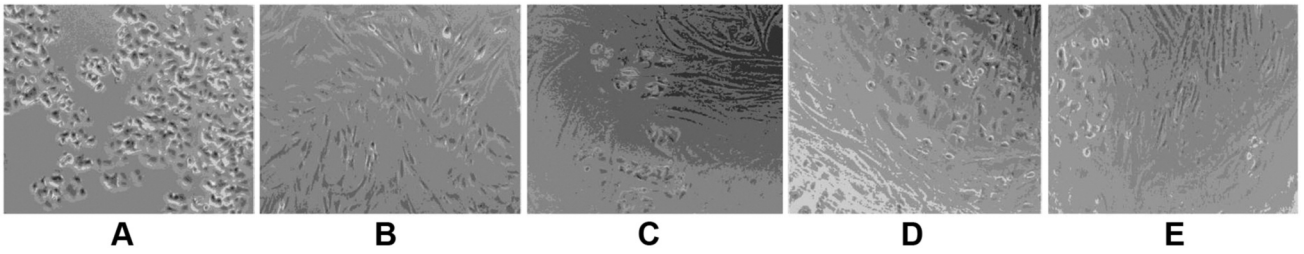

Figure 1. *MCF-7 cells were treated with human bone marrow-derived mesenchymal stem cells (hBM-MSCs) and cell viability examined by light microscopy. A: Group 1: MCF-7 cells cultured alone. B: Group 2: hBM-MSC cells cultured alone. C: MCF-7 cells after 72-h culture with 30,000 hBM-MSCs (30K). E: MCF-7 cells after 72-h culture with 40,000 hBM-MSCs (40K).*

cells. Data clearly suggested that 30% of mice experienced complete remission of the tumor (14).

It was recently convincingly revealed that culturing of placental-derived adherent stromal cells in the presence of tumor necrosis factor-alpha (TNFα) and IFNγ transiently up-regulated the expression of numerous antiproliferative and anti-angiogenic cytokines (15).

MSCs transduced with an adenoviral vector which contained membrane-anchored and secreted TRAIL expression units, including the MUC16 (CA125)-targeted variant Meso64-TR3, have recently been tested for efficacy. Preclinical studies provided evidence that MSC-derived Meso64-TR3 efficiently inhibited tumor growth in mice inoculated with OVCAR3 cells (16).

There is direct evidence to suggest that mature IFN-dendritic cells generated from IFN-dendritic cells primed with OK-432 (streptococcal preparation) significantly enhanced cell-surface expression of TRAIL (17). IFNα and celecoxib synergistically reduced growth of hepatitis B virus-infected HLCZ01 cells. Mechanistically it was shown that IFNα (4,000 IU/ml) increased the level of *TRAIL* mRNA in hepatitis B virus-infected HLCZ01 cells (18).

In the present study, we investigated the apoptotic effects of human bone marrow-derived (hBM) MSCs on breast cancer cell line MCF-7 with IFNγ and determined the cytokines affected in the apoptotic process.

Materials and Methods

Cell lines and culture. hBM-MSCs were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the recommended protocol in Mesenchymal Stem Cell Basal Medium (485 ml; ATCC) supplemented with Bone Marrow-Mesenchymal Stem Cell Growth Kit (ATCC) and antibiotics (Gibco, Grand Island, NY, USA), under mycoplasma-free conditions at 37˚C in a humidified atmosphere with 5% CO_2. For the removal of non-adherent cells, the medium was changed after 2 days. Cell culture medium was changed biweekly, and cells were passaged with 0.25% trypsin 0.1% EDTA (Gibco BRL) until reaching 90% confluency. hBM-MSCs were used in the experiments before the fifth passage.

Human breast cancer cell line MCF-7 was supplied from Department of Biochemistry Medical School of Uludag University and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco) and antibiotics, under mycoplasma-free conditions at 37˚C in a humidified atmosphere with 5% CO_2. For the removal of non-adherent cells, medium was changed after 2 days. Cell culture medium was changed biweekly and cells were passaged with 0.25% trypsin 0.1% EDTA until they attained 90% confluency.

Co-culture of hBM-MSCs with MCF-7 cells. For indirect co-cultures, hBM-MSCs (25,000 cells) were thawed and seeded on a transwell chamber (pore size=0.4 μm; (Nunc™; ThermoFisher, Paisley, UK) in 6-well plates (Nunc™; ThermoFisher) and incubated with or without 0.5 μg/ml human recombinant IFNγ (Merck Millipore, Danville, CA, USA) for 24 h at 37˚C in a humidified atmosphere with 5% CO_2. MCF-7 cells were thawed and seeded on 6 well-plates alone until confluency for 24 h of incubation at 37˚C in a humidified atmosphere with 5% CO_2. After this period, for indirect co-culture experiments, hBM-MSCs on the transwell chamber (upper chamber) were transferred to 6-well plates with MCF-7 cells seeded at 25,000, 50,000, and 75,000 cells/cm^2. For direct co-culture experiments, hBM-MSCs were seeded at 10,000, 20,000, 30,000, 40,000, 50,000 and 70,000 cells/cm^2 with or without 0.5 μg/ml human recombinant IFNγ (Merck Millipore) for 24 h at 37˚C in a humidified atmosphere with 5% CO_2, then MCF-7 cells (50,000 cells) were inoculated on hBM-MSCs and these were incubated at 37˚C in a humidified atmosphere with 5% CO_2 for a further 72 h. hBM-MSCs and MCF-7 cells were also cultured individually as control groups in 500 μl RPMI 1640 containing 10% fetal bovine serum and antibiotics, in 48-well cell culture plate (Nest Biotech, Wuxi, Jiangsu, PR China).

MCF7 cells with hBM-MSCs were cultured directly or indirectly for 72 h under standard culture conditions. Conditioned medium was collected, filtered (0.45 μm), and stored at −80˚C until analysis. After culture, cells were harvested using 0.25% trypsin without EDTA and stained using MSC markers phycoerythrin-conjugated CD73 (BD Biosciences, San Jose, CA, USA) and phycoerythrin-conjugated CD90 (BD Biosciences) for gating out hBM-MSCs and were then analyzed *via* flow cytometry.

Flow cytometric analysis. After co-culture, cells were harvested using 0.25% trypsin without EDTA for apoptosis analysis using CD95-fluorescein isothiocyanate (FAS-FITC) apoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were gated for CD90-positive and

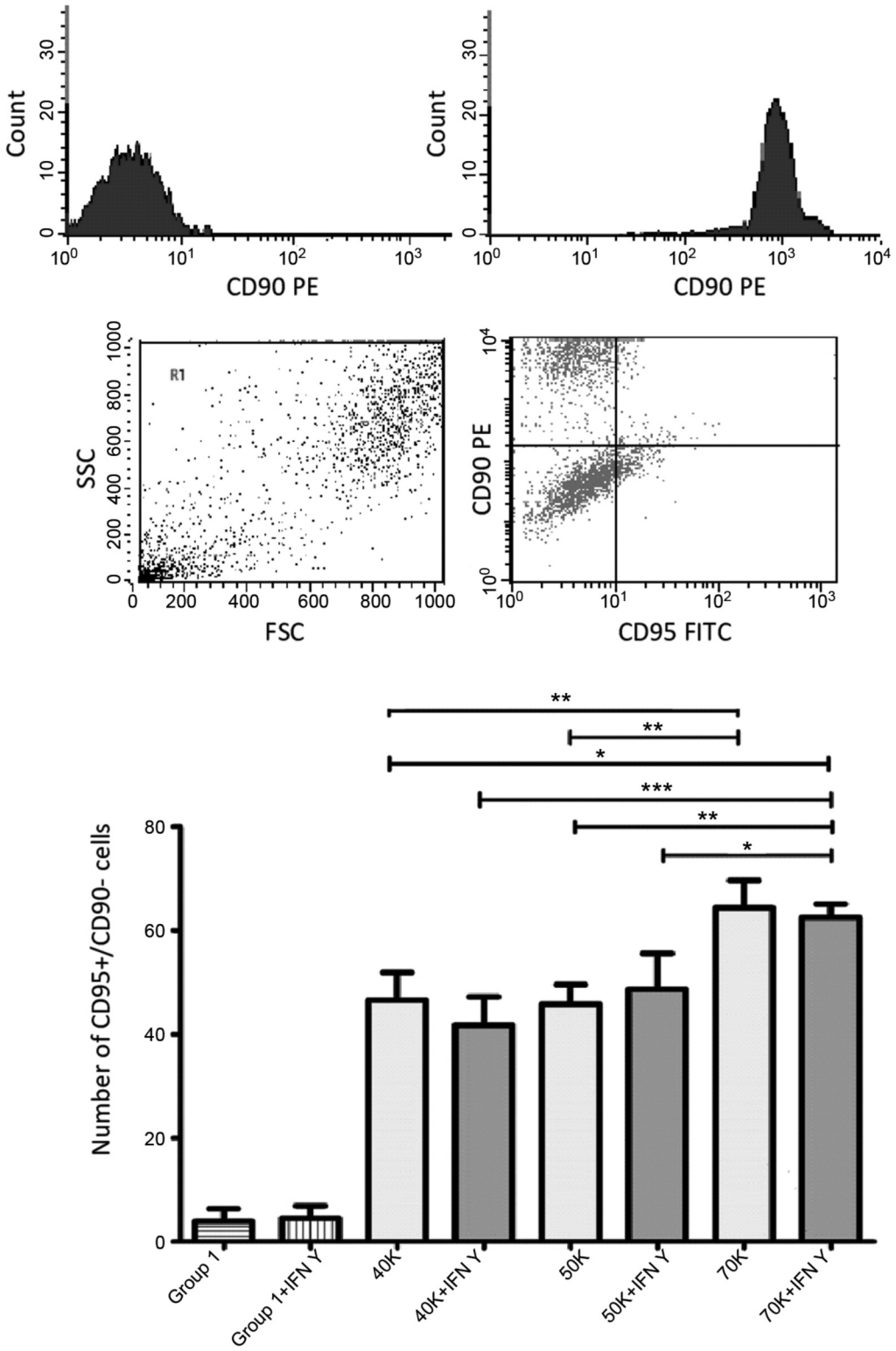

Figure 2. *Growth inhibition of MCF-7 cells by human bone marrow-derived mesenchymal stem cells (hBM-MSCs) in direct culture. Effect of hBM-MSCs as detected by the CD95^{+}/CD90^{-} test. The apoptotic effect of 70,000 (70K) hBM-MSCs on the CD95^{+}/CD90^{-} rate of MCF-7 cells was analyzed by flow cytometry. In the presence of interferon-γ (IFNγ), the apoptotic effect of 70 K hBM-MSC on the CD95^{+}/CD90^{-} rate of MCF-7 was assessed by flow cytometry. Statistical analysis was performed by one-way ANOVA against MCF-7 cells alone (Group 1). Significantly different at *p<0.05, **p<0.01 and ***p<0.001.*

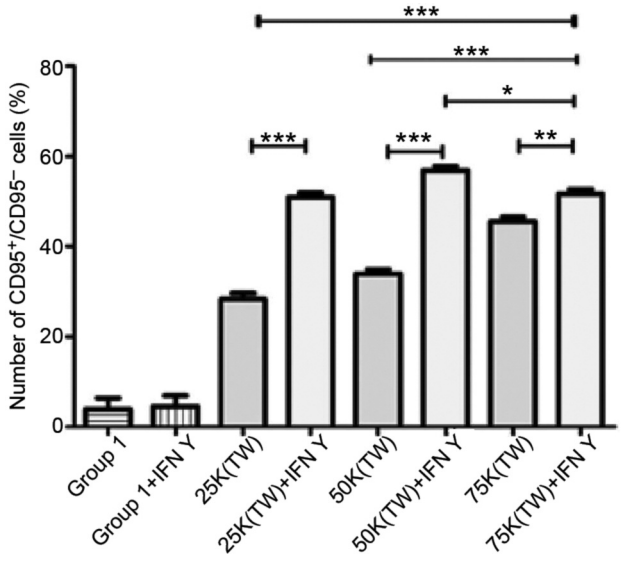

Figure 3. *MCF-7 cell apoptosis in transwell (TW) system with human bone marrow-derived mesenchymal stem cells (hBM-MSCs) as detected by the CD95$^+$/CD90$^-$ test. In the presence of interferon-γ (IFNγ), the apoptotic effect of 70,000 (70K) hBM-MSCs on the CD95$^+$/CD90$^-$ rate of MCF-7 was assessed by flow cytometry. In the presence of IFNγ, the apoptotic effect of hBM-MSCs was higher than in the absence of IFNγ. Group 1: MCF-7 cells cultured alone.Significantly different at *p<0.05, **p<0.01 and ***p<0.001.*

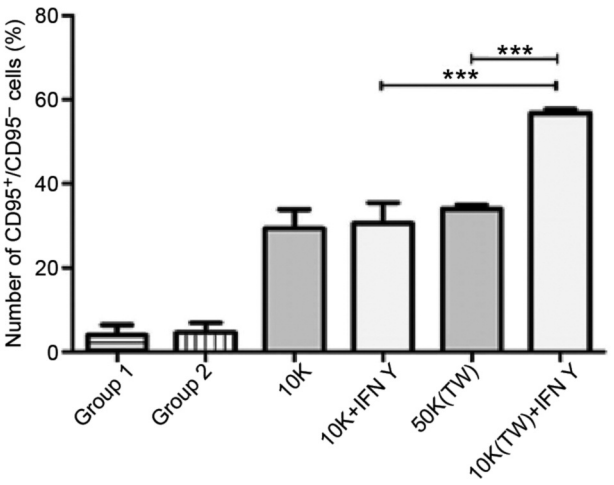

Figure 4. *Comparison of effects of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) on MCF-7 cell apoptosis in transwell system and direct co-culture. In the presence of interferon-γ (IFNγ), the apoptotic effect of 50,000 hBM-MSCs in transwells [50K (TW)] on the CD95$^+$/CD90$^-$ rate of MCF-7 was assessed by flow cytometry. Group 1: MCF-7 cells cultured alone. Group 2: hBM-MSC cells cultured alone. ***Significantly different at p<0.001.*

-negative populations in order to separate hBM-MSCs and MCF7 cells. hBM-MSCs were selected from the CD90-positive area and MCF7 cells from the CD90-negative area. Cell apoptosis was detected by staining CD90 phycoerythrin and FAS-FITC *via* flow cytometry (FACS Calibur; BD Pharmingen).

Enzyme-linked immunosorbent assay. After collection of cell culture supernatants, human TRAIL/APO2L protein levels were analyzed by using commercially available ELISA kit (Diaclone, Besancon, France) according to the manufacturer's instructions and absorbance was measured with Multiskan Spectrum (Thermo Scientific, Waltham, MA, USA).

Statistical analysis. Analysis of the differences between groups was carried out through one-way ANOVA using SPSS v20 (IBM, Armonk, NY, USA) and GraphPad Prism 6 software. Graphs were generated by using GraphPad Prism. *p*-Values of less than 0.05 were considered statistically significant.

Results

hBM-MSC induced apoptosis in MCF7 cells. As IFNs regulate cellular and immune responses, and MSCs home to tumor sites, we evaluated whether hBM-MSCs cultured with IFNγ would affect MCF-7 cell growth or not. hBM-MSCs were cultured with MCF-7 cells for 72 h. Viability of the cells was examined by both light microscopy (Figures 1) and CD95+ ratio *via* flow cytometry (Figure 2). When MCF-7 cells were cultured with hBM-MSCs, there was an increase in the proportion of CD95$^+$ cells beginning at 40,000 hBM-MSCs (p<0.05) (Figure 2). These results suggest that hBM-MSCs increased apoptosis in the MCF-7 cell line in the presence and absence of IFNγ.

To investigate the importance of cell–cell contact in the apoptotic effect of stem cells on the MCF7 cell line, we used a transwell system. Thereafter, the apoptosis of MCF-7 cells was evaluated *via* flow cytometry. When MCF-7 cells were cultured with hBM-MSCs in transwells, there was an increase in the proportion of CD95$^+$ cells beginning at 25,000 hBM-MSCs (p<0.05). In addition, the CD95 expression of MCF-7 cells cultured with hBM-MSCs significantly increased in the presence of IFNγ compared to hBM-MSCs without IFNγ (p<0.001) (Figure 3).

When equal cell ratios were evaluated under direct and transwell systems, the apoptotic effect of hBM-MSCs on the the proportion of CD95$^+$ MCF-7 cells significantly increased in transwell system with IFNγ (p<0.001).

IFNγ in combination with hBM-MSCs increased the level of TRAIL. TRAIL is known to induce cell death in a wide variety of cancer cell lines. Because hBM-MSCs effectively induced MCF-7 apoptosis in a transwell system as well as direct co-culture, we predicted that hBM-MSCs affected MCF-7 apoptosis *via* soluble factors such as TRAIL. To verify this hypothesis, we quantified the level of TRAIL *via*

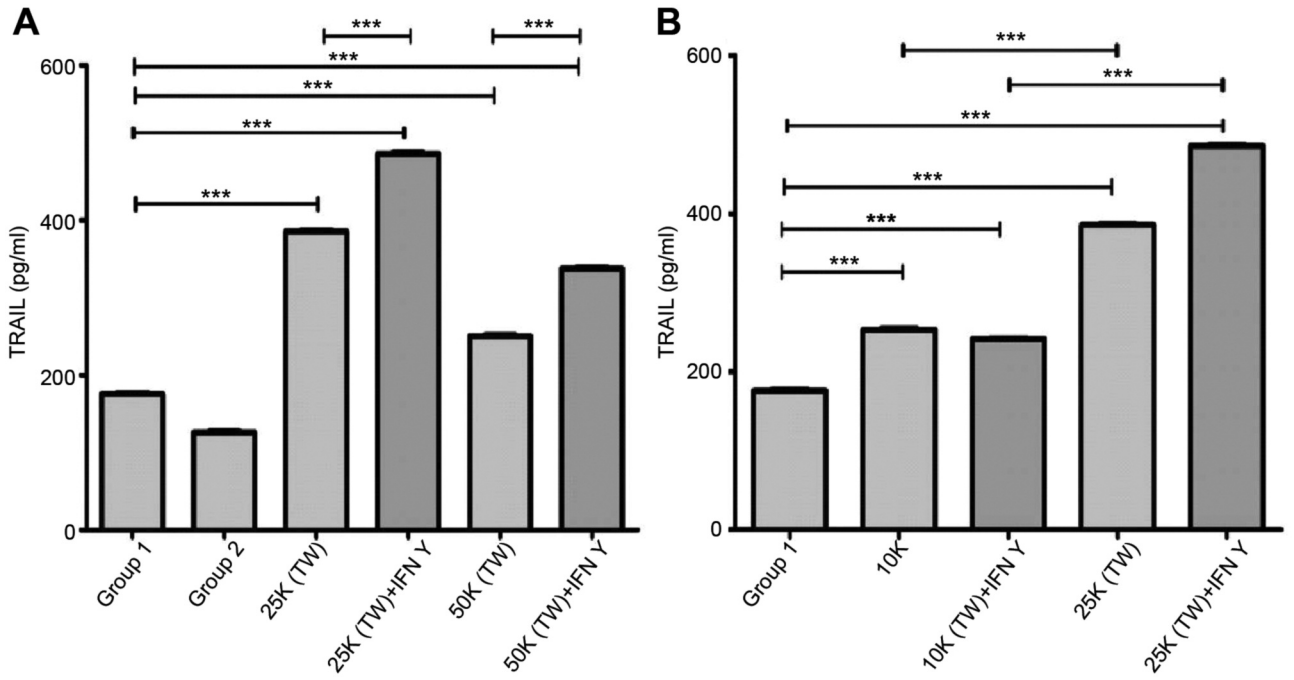

Figure 5. *Effect of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of MCF-7. A: TRAIL levels were determined by enzyme-linked immunosorbent assay. The level of TRAIL produced increased when MCF-7 cells were cultured with 25,000 hBM-MSCs in transwells [25K (TW)]. The TRAIL level increased in the presence of interferon-γ (IFNγ) compared with the absence of IFNγ. B: Comparison of TRAIL levels in the transwell system and direct co-culture. Although the TRAIL level increased in both systems, it was lower in direct co-culture. Group 1: MCF-7 cells cultured alone. Group 2: hBM-MSC cells cultured alone. ***Significantly different at p<0.001.*

ELISA method. The supernatants were collected at 24., 48. and 72. h. After 24 h the level of TRAIL increased in both the direct culture and the transwell system, and the highest level of TRAIL was found after 72-h culture (Figure 4).

MCF-7 cells cultured with hBM-MSCs in transwells expressed significantly more TRAIL compared with MCF-7 cells cultured alone ($p<0.001$). We observed that TRAIL levels were higher when MCF-7 cells were cultured with 25,000 than 50,000 hBM-MSCs (Figure 5A). The presence of IFNγ significantly increased the level of TRAIL expression ($p<0.001$). When cells were cultured at equal ratios in direct culture and transwell systems, the level of TRAIL was significantly higher in the transwell system ($p<0.001$) (Figure 5B).

Discussion

Tumor-homing properties of MSCs have gained significant appreciation. MSCs have showcased a unique ability to contextually regulate tumorigenesis (19, 20). In order to be able to exploit this characteristic in a therapeutic strategy, detailed research into molecular interaction between MSCs and cancer cell lines should be performed and mechanistic insights gained.

The International Society for Cellular Therapy built up the definition of MSCs by taking minimal criteria into account. In this regard, MSCs must be adherent to plastic surfaces in standard culture conditions and must express CD105, CD73, and CD90 cell-surface markers. Due to their high ability for differentiation, much research is being conducted on their differentiation potential. However, in our study, we found that hBM-MSCs did not display any differentiation analyzing CD73 and CD90 markers *via* flow cytometry (data not shown.

Owing to their high proliferative capacity and multilineage differentiation potential, we chose to use hBM-MSCs in the present study. The efficiency and reliability of these cells has been demonstrated by clinical studies. According to Nakazimo *et al*., hBM-MSCs inhibited the growth of glioma cancer cells *in vitro* (21). We aimed to examine the effects of hBM-MSCs on MCF-7 breast cancer cell line comparatively. As a result of our study, we found that hBM-MSCs induced MCF-7 cell apoptosis effectively.

In the present study, MCF-7 cells were direct and indirectly cultured with hBM-MSCs. The transwell system and direct co-culture were studied synchronously and the results were compared. When equal cell rates of direct culture and

transwell system were evaluated, the apoptotic effect of hBM-MSCs as shown by the proportion of $CD95^+$ MCF-7 cells was significantly higher in the transwell system as detected by flow cytometry. These results underline that the ratio of hBM-MSCs to cancer cells in culture has a great importance in observing the apoptotic effect on breast cancer cells.

TRAIL, a pro-apoptotic molecule, induces apoptosis of cancer cells without affecting healthy cells. TRAIL levels were quantified by sandwich ELISA after cells were cultured at equal rates in direct culture and transwell system: the TRAIL level was significantly higher in the transwell system. These findings were similar to the $CD95^+$ findings.

Researchers investigating the effects of IFNs on cancer cells concentrated on IFNα and IFNβ as their antitumor effects are known (22). Yang *et al*. reported that IFNγ-secreting MSCs selectively induced apoptosis in cancer cells but this process was not examined in normal cells (23). Likewise, in the present study, the apoptotic effect of IFNγ on MCF-7 cells was determined *in vitro*. It was observed that the treatment with exogenous IFNγ induced MCF-7 cell apoptosis at a higher rate in co-culture with hBM-MSCs.

Conclusion

hBM-MSCs had an inhibitory effect on MCF-7 cell proliferation, dependent on their proportion and time. This inhibitory effect increased in the presence of IFNγ. The use of hBM-MSCs with IFNγ is promising as a novel therapy for the treatment of breast cancer.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

ENY: Study design and analysis. DG: Study design, analysis and interpretation of results. AAF: Interpretation of results. ST: Analysis and interpretation of results. UZ: Interpretation of results. TA: Interpretation of results. IY: Study design and Interpretation of results.

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