Research Article

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The confounding effect of interleukin-6 on apoptosis of MCF-7 cells through down-regulation of MMP-2/-9 mRNA expression

İnterlökin-6'nın MMP-2/-9 mRNA Ekspresyonunun Downregülasyonu Yoluyla MCF-7 Hücrelerinin Apoptozisi Üzerindeki Etkisi

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Abstract

Objectives: Breast cancer is the second cause of death among women worldwide. In the last decades, the immunotherapy-based approaches have a growing importance in the treatment of breast cancer. Several studies have indicated the pleiotrophic effect of Interleukin-6 (IL-6) via targeting the membrane-bound or soluble receptors.

Materials and methods: Different concentrations of IL-6 were incubated for 24, 48, and 72 h in the breast carcinoma cell line (MCF-7). Cell proliferation, apoptotic cell population, gene expression by RT-PCR were measured, and the effect of IL-6 treatment on cell morphology was observed.

Results: In the present study, IL-6 treatment of MCF-7 cells inhibited cell proliferation in a dose and time dependent manner. The IL-6 treatment was found most effective on 24 h. The viable cell amount was decreased to $70.07 \pm 4.85\%$ at 100 nM treatment with a significant alteration on cell morphology, simultaneously in the 24 h of treatment. IL-6 treatment has also increased the early apoptotic cell population % in MCF-7 cells significantly

($p < 0.0001$). The RT-PCR analyses have shown that the apoptotic effect of IL-6 was related to the decrease at MMP-2/-9 mRNA levels ($p < 0.0001$).

Conclusions: In conclusion, IL-6 treatment may inhibit cell proliferation and induce apoptosis of MCF-7 cells in a dose-dependent manner through down-regulation of MMP-2/-9.

Keywords: apoptosis; interleukin-6; MCF-7; MMP-2/-9; proliferation.

Öz

Amaç: Meme kanseri, dünya çapında kadınlar arasında ikinci ölüm nedenidir. Son yıllarda, meme kanserinin tedavisinde immünoterapi temelli yaklaşımların önemi giderek artmaktadır. Çeşitli çalışmalar, interlökin-6'nın (IL-6), membrana bağlı veya çözünebilir reseptörleri hedef alarak pleiotropik etki gösterdiğini belirtmiştir.

Gereç ve Yöntem: Farklı konsantrasyonlarda IL-6 (10, 25, 50, ve 100 nM) meme kanseri hücre hattında (MCF-7) 24, 48 ve 72 saat inkübe edilmiştir. İnkübasyondan sonra hücre proliferasyonu, apoptotik hücre popülasyonu, RT-PCR ile gen ekspresyonu ölçülmüş ve IL-6 tedavisinin hücre morfolojisine etkisi gözlemlenmiştir.

Sonuç: Mevcut çalışmada, MCF-7 hücrelerinde IL-6 tedavisi, hücre proliferasyonunu doza ve zamana bağımlı bir şekilde inhibe etmiştir. 24 saat inkübasyon sonrasında 100 nM IL-6 tedavisi ile canlı hücre miktarı $70.07 \pm 4.85\%$ kadar azalmış, aynı zamanda hücre morfolojisinde anlamlı şekilde değişiklik gözlenmiştir. IL-6 tedavisi ayrıca MCF-7 hücrelerinde erken apoptotik hücre popülasyonu yüzdesini önemli ölçüde artırmıştır ($p < 0.0001$). RT-PCR analizleri, IL-6'nın apoptotik etkisinin MMP-2/-9 mRNA seviyelerindeki düşüşle ilişkili olduğunu göstermiştir ($p < 0.0001$).

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Tartışma: Sonuç olarak IL-6 tedavisi, MMP-2/-9'un downregülasyonu yoluyla doza bağımlı bir şekilde hücre proliferasyonunu inhibe edebilmekte ve MCF-7 meme karsinom hücrelerinin apoptozunu indükleyebilmektedir.

Introduction

Immunotherapy is a form of treatment that modulates the immune system and has been suggested as a potentially powerful approach for the treatment of diseases for a long time [1]. This process is based on the protection of the immune system and, it has developed in the clinical and pre-clinical sense for the treatment of diseases such as transplantation rejection, allergy, infectious diseases, autoimmunity as well [2, 3].

Immunotherapy has been shown to be a promising approach for recurrent and metastasized cancers [4]. In this context, the use of monoclonal antibodies to prevent the activity of immune control points, activation of the anti-cancer T cell response, and the attacking of the patient's own lymphocytes into cancer cells by adoptive cellular therapy are examples of immunotherapy-based studies [5].

In recent years, steady advances in basic cancer immunology and translational immunotherapy have led to the development of two classes of treatment approaches that are significantly effective in cancer patients. One of them is Adoptive cell therapy (ACT), which is mainly based on the injection of autologous tumor-targeted-T cells [6, 7]. The other is the control point blockade based on the inhibition of the inhibitor cytotoxic T lymphocyte antigen-4 or programmed death 1 receptors [8, 9]. The researches have indicated that ACT treatment completely responds to advanced metastatic melanoma and several hematological cancers. This is thought to be related to the complete elimination of detectable tumor burden, and some of these are also long-lasting permanent responses [10].

In a typical immune response, antigens are captured by dendritic cells (DCs), which then mature and deliver the antigenic peptide to T cells in the lymph nodes in the context of major histocompatibility complex (MHC) molecules. Thus, effector T cells are produced to migrate to areas of infection, inflammation, or damage. Eventually, DCs release cytokines such as IL-1 β , IL-6, IL-12, and tumor necrosis factor (TNF), which shape the natural killer cell and T cell responses [11].

While Interleukin-6 (IL-6) is known as the leading inflammatory cytokine, it also acts as an anti-inflammatory myokine. Based on these two-directional effects of IL-6, different studies have shown that effective results can be obtained against cancer by IL-6 application or inhibition of IL-6 receptor [12–15]. The researches have revealed that IL-6 shows its primary anti-tumor function through the

promotion of anti-tumor immunity [16]. Since IL-6 signaling is known to initiate the T cell immune responses [17], it's accepted as a key cytokine in the management of anti-tumor immune response [18].

In the light of these considerations, the present work aimed to evaluate the effect of IL-6 treatment on apoptosis of MCF-7 estrogen-positive human breast cancer cells.

Materials and methods

Cell culture

Human breast cancer MCF-7 cells obtained from the American Type Culture Collection (Germany) were cultured in DMEM (Sigma, Germany) supplemented with 10% fetal bovine serum (Sigma, Germany) and 1% penicillin–streptomycin (Sigma, Germany). Cells were grown in a humidified atmosphere in 5% CO₂ and 37 °C. IL-6 (eBioscience, USA) was dissolved in the diluent buffer and diluted at a concentration range between 10 and 100 nM.

Cell proliferation assay

Cell proliferation was determined by *in vitro* MTT assay, according to Mossman [19]. Briefly, the cells were seeded at a density of 1×10^4 cells/well and then treated with 10, 25, 50, and 100 nM IL-6 solutions for 24, 48, and 72 h. Following incubation, a 20 μ L of 5 mg/mL MTT solution (Sigma, Germany) was added to all wells and incubated at 37 °C for 2 h. The absorbance at 540 nm was recorded with a microplate reader (Thermo, Germany). The data represent the mean $\pm$ standard deviation (SD) of two independent experiments.

Morphological observation

5×10^4 /mL suspension of MCF-7 cells were seeded in 96-well plates at 180 μ L per well and cultured overnight until the cells adhered to the wall. The cells were exposed to IL-6 (100 nM) for 24, 48, and 72 h and the same volume of the consumption of diluent buffer for solubilizing IL-6 was considered a control group. The morphological changes of MCF-7 cells were observed and photographed under an inverted phase-contrast microscope (Leica, Germany).

Annexin V binding

The cells were plated into six-well plates at a density of 1×10^6 cells/well and incubated for 24 h. The cells were then treated with IL-6 at 50 and 100 nM concentrations. Following incubation, the cells were harvested, centrifuged, and resuspended in DMEM medium. Annexin V assay kit (Millipore, Germany) was performed according to the manufacturer's instructions. Briefly, 100 μ L of Annexin V reagent was added to 100 μ L of cell suspension and incubated at room temperature for 20 min. Then, the apoptotic cell population was detected by Muse Cell Analyzer (Millipore). The apoptotic cells were determined by the Annexin V positivity based on phosphatidylserine exposure, and dead cells were determined by the nuclear dye 7-AAD (7 aminoactinomycin D)

positivity. Four different cell population were enabled to examine by cytofluorometric separation on a Muse cell analyzer: non-apoptotic live (lower left [LL]: 7-AAD negative, apoptosis negative), non-apoptotic dead (upper left [UL]: 7-AAD positive, apoptosis negative), apoptotic live (lower right [LR]: 7-AAD negative, apoptosis-positive), and apoptotic dead (upper right [UR]: 7-AAD positive, apoptosis-positive) cells.

RT-PCR analysis

MCF-7 cells were treated with IL-6, and total cellular RNA was extracted using RNEasy plus mini kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was synthesized from isolated total RNA for each sample and used for PCR amplification. For real-time Polymerase Chain Reaction (RT-PCR) analysis, reactions were performed in duplicate using Rotor Gene System (Qiagen). RT2 qPCR Primer Assay numbers for Matrix metalloproteinase-2 (MMP-2), MMP-9, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes were PPH00151B, PPH00152E, and PPH14985F, respectively (Qiagen, Germany). The data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method calculating the difference between the threshold cycle (Ct) values of the target and reference gene of each sample.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0 version (GraphPad Software Inc.). Data obtained from the cell culture experiments were expressed as mean $\pm$ SD, and a one-way Analysis of Variance (ANOVA) test was applied for multiple comparisons.

Results and discussion

The goal of immunotherapy is to produce a strong immune response, thereby stimulating cytotoxic lymphocytes to destroy tumor cells and eventually acquiring a long-term anti-cancer immunity. The relationship between the immune system and cancer has been confirmed by the findings of which the immune system strongly regulates the anti-tumor effect. The response of the immune system around the tumor microenvironment and inflammatory processes can provide tumor-promoting signals, and the immune response remains a target for treating cancer due to the natural target specificity, adaptability, and potential for permanent protection of the immune system [20]. IL-6, one of the many cytokines released by the immune system, has a pleiotropic effect by promoting cancer formation and helping the development of anti-tumor immunity by activating T cell responses [21].

The effect of IL-6 is determined by signaling pathways activated by binding to membrane-bound (mIL-6R α) and soluble receptors (sIL-6R) [22]. The trans-signaling pathway in which IL-6 binds to sIL-6R [23], modulates the response of T cells and causes the induction of apoptosis as a pro-inflammatory effect [17, 24]. The pleiotropic effect of IL-6 on cancer cells were figured out (Figure 1).

Figure 1: IL-6 signaling pathways. In the classical pathway, after IL-6 binds to membrane-bound IL-6 receptor alpha (mIL-6R α), this complex interacts with the signal-transducing membrane glycoprotein 130 (gp130), and the gp130 becomes dimerized. In the trans-signaling pathway, IL-6 first binds soluble IL-6R (sIL-6R), then combines with the complex gp130. For both signaling paths, the activated gp130 triggers the Janus Kinase/Signal transducer and activator of transcription-3 (JAK/STAT3) pathway. Phosphorylated STAT3 goes to the nucleus and initiates cancer formation, activates apoptosis, and decreases MMP levels relative to the cytokines released due to the classical or trans-signal pathway. In the classical pathway, both the gp130 and IL-6 receptors are membrane-bound, and therefore only classical signaling is seen in cells with mIL-6-R α . IL-6 trans-signaling can occur in any cell type with membrane-bound gp130; therefore, trans-signaling is thought to be capable of activating more gp130 signal transducers compared to classical IL-6 signaling.

The studies have reported that IL-6 and sIL-6R complex blocks the cell cycle in the G1 phase by inhibiting cell proliferation in various cell lines such as melanoma [25, 26], breast cancer [27], and osteosarcoma [28]. It is believed that this growth suppression and cytostatic effect occur by activation of STAT21 and stimulation of cell cycle inhibitors such as p21WAF1 and/or p27KIP1 [29].

In the present study, in order to evaluate the effect of IL-6 on the proliferation of MCF-7 cells, the cells were treated with 10, 25, 50, and 100 nM of IL-6 for 24, 48, and 72 h, and the cell viability was determined by MTT assay. It's been observed that the growth inhibitory effect of IL-6 on MCF-7 cells was manifested in a concentration-dependent manner. The results showed that the cell proliferation significantly decreased at 25 nM and higher concentrations, and the viable cell amount decreased to $70.07 \pm 4.85\%$ at 100 nM treated group when compared to control in 24 h incubation. On the other hand, the viable cell amount were 78.61 ± 1.24 and $82.41 \pm 2.91\%$ in 48 and 72 h incubation, respectively. The IC_{50} values were calculated as 157.99, 267.45, and 294.05 nM in a time dependent manner and the results revealed that IL-6 inhibited the proliferation of MCF-7 cells more intensely at 24 h when compared to other time points (Figure 2).

The alteration on MCF-7 cell morphology by the treatment with IL-6 at different time points were investigated by an inverted microscope. In the control group, the average breast adenocarcinoma cells were in close contact with each other and saved their polygonal shapes, while 100 nM IL-6 treated cells loosed the specific shapes through forming aggregates (Figure 3). Additionally, the cellular intensity was lower at 24 h incubation when compared to 48 and 72 h indicating that the antiproliferative effect of IL-6 treatment decreases depending the application time.

Figure 2: The effect of IL-6 on cell proliferation of MCF-7 breast adenocarcinoma cells. The cells were treated with 10, 25, 50, and 100 nM IL-6 for 24, 48, and 72 h and cell viability was evaluated by MTT assay. Results are expressed as a percentage of viable cell amount. The nontreated cells were used as control. Each value represents the mean $\pm$ standard deviation from three independent experiments performed in triplicate (* $p < 0.01$, # $p < 0.0001$, compared to control).

High serum and tissue expression of tissue inhibitors of metalloproteinase-1 (TIMP-1) is related to poor prognosis and reduced survival for many cancers. Since TIMPs can restrain MMP-associated activities, they are accepted as tumor-inhibitory factors [30].

In our study, the MCF-7 cells were treated with 50, and 100 nM of IL-6 for 24 h, and the apoptosis was determined via Annexin V binding to the cell membranes. The results showed that the early apoptotic cell population % was significantly increased to 8.82 ± 1.28 and $12.36 \pm 0.89\%$ in 50 and 100 nM treated groups, respectively ($p < 0.01$, $p < 0.0001$) when compared to control (Figure 4). The increase in the apoptotic cell population were reverse compatible with the decrease in live cell population.

Based on the knowledge that IL-6 shows pro-inflammatory activity depending on the type of receptor to which it binds, and it's associated with a decrease in MMP levels, we evaluated the effect of IL-6 on MMP-2/-9 mRNA expression of MCF-7 cells by RT-PCR experiments. The cells were treated with 100 nM of IL-6 for 24 h, and the fold change of mRNA levels were calculated by $2^{-\Delta\Delta Ct}$ method. The results showed that the IL-6 treatment resulted in 0.124 and 0.482 fold-decrease at MMP-2 and MMP-9 mRNA expression levels, respectively when compared to control ($p < 0.0001$) (Figure 5). This method is capable of calculating the quantitative gene expression levels by collecting the results after the reaction is complete without any requirement like traditional methods. Thus, the alteration on MMP levels could be explained as only with a fold-change value.

The pleiotropic effects of IL-6 have been identified in different cell lines. Chiu et al. [31] have reported that the administration of IL-6 reduced the proliferation of MCF-7 cells and induced apoptosis through DNA fragmentation. Wang et al. [32] observed that cell growth of LNCaP cells decreased significantly with IL-6 treatment. Zhuang et al. [33] have demonstrated the apoptotic effect of IL-6 on hepatocarcinoma cells, without any inhibitory activity on proliferation and invasion of cells. Zhang et al. [34] showed that IL-6 reduced MMP-10 mRNA levels but increased the MMP-10 protein mass in A549 lung carcinoma cells. On the other hand, Sun et al. [35] showed that 2-hydroxy-3-methylantraquinone reduces cell proliferation by downregulating the IL-6-mediated JAK2/STAT3 pathway in lung cancer cells.

Conclusion

This study has been focused on the pro-inflammatory effect of IL-6 on MCF-7 breast adenocarcinoma cells. The results

Figure 3: Morphology of breast adenocarcinoma MCF-7 cells under inverted phase-contrast microscopy at different time points. Morphologically, IL-6 (100 nM) administration induced a significant cell rounding and growth inhibition at the 24 h. Dilution buffer administered well was recorded as control (magnification 12.6×).

Figure 4: The results of the Annexin V binding assay. The MCF-7 cells were treated with 50 and 100 nM IL-6 for 24 h, and the apoptosis was detected by the Muse cell analyzer (Millipore). Nontreated cells were used as control. Four populations of cells can be distinguished in this assay: non-apoptotic cells: Annexin V (–) and 7-AAD (–); early apoptotic cells: Annexin V (+) and 7-AAD (–); late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+); mostly nuclear debris: Annexin V (–) and 7-AAD (+). The results were given for three independent experiments, and the differences are * $p < 0.01$, # $p < 0.0001$ from control.

indicated that IL-6 inhibited cell proliferation in a dose-dependent manner and also induced apoptosis of cells through increasing the exposure of phosphatidylserine outside of the cell membrane. The effect of IL-6 on MMP-2/-9 mRNA expression has been studied for the first time, and our results showed that IL-6 down-regulated the expression of related genes. Although studies on the effects of

immunotherapy-based treatment modalities in cancer are gaining importance, there is no data in the literature regarding the efficiency of IL-6 administration on breast cancer, which is the second most common type of cancer in women worldwide. This study is the first to demonstrate both the antiproliferative effect of IL-6 treatment and its down-regulatory effect on MMP-2 and MMP-9 genes, which

Figure 5: The effect of IL-6 (100 nM) on mRNA expression of MMP-2 and MMP-9. $\Delta\Delta C_t$ method was used to determine the relative mRNA expression of MMP-2/-9 genes in MCF-7 cells. Mean $\pm$ SD are shown (n=3). The differences are * and # from control: $p < 0.01$ and $p < 0.0001$, respectively.

are known to play an important role in cancer metastasis. In conclusion, the findings showed that IL-6 shows its pro-inflammatory effects on MCF-7 cells in a dose-dependent manner, and this effect could be related to the sIL-6R binding on the membrane. Further studies are required to identify this mechanism of effect.

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