Research Article

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Title: The role of Odoroside A in immune transformation in RAW264.7 cells.

Short title: Effect of Odoroside A on macrophages.

Abstract

Purpose: Macrophages are one of the most important elements of the immune system and play dual role in inflammation. They regulate both the formation of the immune response in the environment and the suppression. This signaling in the cancer microenvironment affects the progression of cancer. This cancer-promoting or cancer-eliminating process is managed by macrophages. The increase in pro-inflammatory cytokine genes provides suppression of tumor cells in the environment. It was aimed to investigate the effect of Odoroside A on macrophage transformation.
Materials and methods: The non-lethal dose of Odoroside A in macrophage cells was determined by CCK-8 analysis. Then, gene expression analysis of cytokines genes and surface markers in RAW264.7 cells was performed by using qRT-PCR. The protein levels of the cytokines that were significant in the gene expression analysis were examined using the ELISA method.
Results: Odoroside A significantly increased the expression of IL1 and IL6 genes in RAW264.7 cells, while it significantly decreased the expression of IL10 gene. There was no significant change in IL4 and TGFβ gene expressions. IL1 and IL6 gene expressions, which had a statistically significant increase, were also found to increase significantly at the protein level.
Conclusions: When all the data are evaluated together, it has been observed that Odoroside A increases the pro-inflammatory response in RAW264.7 cells, thus providing M1 type macrophage transformation. This suggests that Odoroside A may be effective in tumor elimination of the macrophage-mediated environment in the tumor niche.
Keywords: Odoroside A, macrophage, cytokines.

Makale başlığı: RAW264.7 hücrelerinde Odoroside A'nın immün transformasyondaki rolü.

Kısa başlık: Odoroside A'nın makrofajlar üzerine etkisi.

Öz

Amaç: Makrofajlar bağışıklık sisteminin en önemli unsurlarından biridir ve inflamasyonda ikili rol oynamaktadır. Hem ortamda immün yanıtın oluşumunu hem de baskılanmasını düzenlemektedir. Kanser mikroçevresindeki bu sinyalleme, kanserin ilerlemesini etkiler. Bu kanseri teşvik eden veya kanseri ortadan kaldıran süreç makrofajlar tarafından yönetilmektedir. Proinflamatuar sitokin genlerinin ifadesindeki artış, ortamdaki tümör hücrelerinin baskılanmasını sağlar. Bu çalışmada Odoroside A'nın makrofaj dönüşümüne etkisinin araştırılması amaçlanmıştır.

Gereç ve yöntem: Makrofaj hücrelerinde öldürücü olmayan Odoroside A dozu CCK-8 analizi ile belirlenmiştir. Daha sonra RAW264.7 hücrelerinde sitokin genlerinin ve yüzey belirteçlerinin qRT-PCR kullanılarak gen ekspresyon analizi yapılmıştır. Gen ekspresyonu analizinde anlamlı çıkan sitokinlerin protein düzeyleri ELISA yöntemi kullanılarak incelenmiştir.

Bulgular: Odoroside A, RAW264.7 hücrelerinde IL1 ve IL6 genlerinin ekspresyonunu önemli ölçüde artırırken, IL10 geninin ekspresyonunu önemli ölçüde azaltmıştır. IL4 ve TGFβ gen ifadelerinde anlamlı bir değişiklik olmamıştır. İstatistiksel olarak anlamlı artış gösteren IL1 ve IL6 gen ekspresyonlarının protein düzeyinde de anlamlı düzeyde arttığı belirlenmiştir.

Sonuç: Tüm veriler birlikte değerlendirildiğinde Odoroside A'nın RAW264.7 hücrelerinde proinflamatuar yanıtı arttırdığı, dolayısıyla M1 tip makrofaj dönüşümünü sağladığı gözlenmiştir. Bu, Odoroside A'nın, tümör nişindeki makrofaj aracılı oluşan proinflamatuar ortamın tümörün ortadan kaldırılmasında etkili olabileceğini düşündürmektedir.

Anahtar kelimeler: Odoroside A, makrofaj, sitokinler.

Introduction

Macrophages are the first cell group to respond among immune cells. Macrophages can be classified as classically activated M1 (pro-inflammatory) or alternatively activated M2 (anti-inflammatory) phenotypes based on their pro- and anti-inflammatory functions under various stimuli [1]. M1 macrophages play a key role in the positive immune response by presenting antigens, secreting pro-inflammatory cytokines and chemokines, and acting as immune monitors. The primary pro-inflammatory cytokines that are produced by it are TNFα, IL6, and IL12. M2 macrophages secrete a variety of anti-inflammatory cytokines, including TGF-β, IL10, and Arginase-I. These cytokines can reduce inflammation, promote tumor growth, and have an immune-suppressive effect [2]. It is crucial for tissue repair and wound healing. It's interesting to note that these pro- and anti-inflammatory mechanisms could interact. Because injured areas are frequently targets of pathogen invasion, eradicating damage necessitates a balance between macrophage types. As a result, both functioning macrophages and macrophages that fall outside of the M1 and M2 spectrum can be found in these situations [3]. Through the recruitment of pro-immunostimulatory leukocytes and the phagocytosis of tumor cells, M1 cells initiate the production of cytokines inside the tumor microenvironment (TME) and aid in the killing of tumor cells. Nonetheless, other research indicates that M2 macrophages play a crucial part in the dissemination of tumors [4]. According to studies by Quail and Joyce [5], M2 cells have been demonstrated to promote the growth of tumors in both primary and metastatic locations by interfering with the formation and accumulation of basement membranes, angiogenesis, the recruitment of leukocytes, and general immunosuppression.

Cardiac glycosides, which are classified into A and B types, are a class of chemicals having a steroid-like structure that are widely found in angiosperms. They are also referred to as cardiotonic or cardiac glycosides. Apocynaceae (Oleaceae oleander, Scutellaria), Rosaceae (Pygnus, milkweed), and Liliaceae (Lily of the Valley, evergreen) are the families in which type A is primarily found, whereas B type is primarily found in Liliaceae (scallop) and Ranunculaceae [6]. Nerium oleander Linn., a traditional folk remedy, contains an active cardenolide called odoroside A (OA) (Apocynaceae). Due to their cardiovascular toxicity, clinically utilized cardiac glycosides have a narrow therapeutic index, which may restrict their use in therapy [7, 8]. The solution to this issue is to look for chemicals derived from cardiac glycosides that can stop cancer cells from proliferating and spreading without endangering the heart. According to *in vitro* research, cardiac glycosides are safe for normal cells at nanomolar doses and may even prevent apoptosis or promote cell growth in them; however, in cancer cells, these medications

inhibit cell growth and cause cell death [9-11]. According to previous research, OA reduced cell activity in human cancer cell lines, such as those from the stomach, colon, and cervical regions [12]. Mono glycosidic cardenolides, such as oleandrin and OA that were isolated from Nerium oleander, have been shown to have strong anticancer properties [13]. The effects of OA and the underlying processes behind them are less understood than those of oleandrin.

When using anticancer treatments, not only cancer cells but all cell types in that microenvironment are affected. Studies have shown that M2 type macrophages, which are especially abundant in the tumor microenvironment, are tumor promoters. The pro or anti-inflammatory effect created by macrophage transformation in the tumor microenvironment is very critical for tumor development or elimination. The aim of this study is to show how OA affects the transformation in macrophage cells.

Materials and methods

Chemicals

Odoroside A (Merck), CCK-8 kit (Abbkine), DMEM (Gibco), fetal bovine serum (FBS) (Capricorn Scientific), PBS (Biological Industries), penicilin/streptomycin (Biological Industries), QIAzol (Qiagen), cDNA kit (Bio-Rad) and qRT-PCR mix (Solis BioDyne) commercially obtained.

Cell culture

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC TIB-71™). Proliferation of the murine macrophage cell line RAW 264.7 was achieved in the appropriate culture medium using DMEM, 10% FBS, 2 mM L-glutamine, 1% Penicillin-Streptomycin. Cell proliferation, passage and follow-up processes were monitored with an inverted microscope. It was incubated in an oven with 95% humidity and 5% CO_2 until sufficient growth was achieved.

CCK-8 proliferation analysis

CCK-8 colorimetric experiment was conducted to determine non-toxic doses of OA in RAW264.7 cells. DMSO was used to dissolve OA. In a 96-well plate, $1x10^3$ cells/well were used to seed cells. For 24 and 48 hours, the cells were exposed to OA at different concentrations (100, 200, 500, 1000, 2000, 3000, and 4000 nM). Then, using a microplate reader, levels of cell viabilities were measured at 450 nm to calculate cell viability (%).

Gene expression analysis by qRT-PCR

Following the instructions provided by the manufacturers, QIAzol and the Transcriptor First-Strand cDNA Synthesis Kit were used to isolate total RNA from

RAW264.7 cells and synthesize cDNA, respectively. IDT PrimerQuest was used to build the primer sequences for the genes involved in this study's qRT-PCR analysis, and the results were shown in Table 1 [14]. For every gene, a qRT-PCR reaction mix was made. In summary, each reaction contained 4 µl of Solis qRT-PCR master mix, 5 pmol of forward and reverse primer, and 2 µl of cDNA. The steps in the qRT-PCR technique were as follows: 12 minutes of initial denaturation at 95°C, 15 seconds of denaturation at 95°C, 20 seconds of annealing at 60°C, and 20 seconds of extension at 72°C. The PCR reaction was run for 40 cycles. Utilizing the Bio-Rad CFX Connect™ Real-Time System, qRT-PCR analysis was carried out. Melting curve analysis was performed by gradually heating the PCR products from 65°C to 95°C. In the study, GAPDH was used as the reference gene.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were extracted from cell cultures. To measure the protein amounts of IL1 and IL6, SunRedBio ELISA kits (Shanghai SunRed Biological Technologies, China) were utilized. After centrifuging the supernatants for 20 minutes at 3000 rpm, 10 µL of samples and 40 µL of dilution buffer were put to 96-well plates. The wells were rinsed five times for 30 seconds each after being incubated for 30 minutes at 37 °C. Each well received 50 µL of HRP-conjugated reagent, which was then incubated for 30 minutes at 37°C. Following the cleaning, each well was filled with 50 µL of chromogen solutions A and B, and the wells were gently shaken. 50 µL of stop solution was added to end the reaction after it had been incubated for 15 minutes at 37°C in the dark. At 450 nm OD, absorbance was determined with an ELISA reader (Epoch, USA).

Statistical analysis

Using the $2^{(-\Delta\Delta Ct)}$ technique, the Ct values of the genes examined in the study were standardized with respect to the reference gene. The GraphPad®Prism version 9.2.0 software was used to assess the groups' cytokine quantities and gene expression levels using the "Multiple t test." Statistics were deemed significant if $p<0.05$.

The study is a cell culture study that does not require ethics committee approval.

Results

In the viability test of OA in RAW264.7 cells, the selected doses were shown to be non-toxic (Figure 1). Since the aim of this study was to see how OA affects cellular transformation in macrophage cells rather than the lethal dose, a dose of 500 nM was used for 24 h.

Expression analysis of OA in inflammatory genes is shown in figure 2. According to gene expression analysis, OA caused a significant increase in IL1 and IL6 genes and

significant decrease in IL10 gene in RAW264.7 cells, while it caused a non significant changes in IL4 and TGFβ.

The expression levels of CD86 and CD163 markers, which are important markers in the transformation of macrophage cells, are shown in figure 3. Accordingly, while CD86 expression, which is a pro-inflammatory change, increased significantly, CD163 expression decreased significantly.

ELISA analysis was performed to show how gene expression analyzes change at the protein level. The data obtained according to ELISA analysis were found to be compatible with gene expression analysis (Figure 4).

Discussion

Macrophages play a significant part in the control of physiological processes and are found in many tissues devoid of blood vessels [15]. They are useful in the initiation, maintenance, and termination of the inflammatory response because of their great elasticity [16, 17]. Tissue integrity is not ideal when pro-inflammatory cytokines are released in large quantities. According to Gordon and Martinez [18], macrophages aid in the inhibition of the inflammatory response. Tissue purification and the restoration of typical physiological circumstances take place [19]. It is commonly recognized that chronic inflammation is linked to the majority of malignancies (90-95%). As a result, M2 macrophages can promote the growth of tumors, while M1 macrophages can contribute to a mutagenic microenvironment [20, 21]. The role of macrophages in the tumor microenvironment is noteworthy. They are the most effective elements in creating a pro-inflammatory or anti-inflammatory environment in the tumor microenvironment. In addition to the effectiveness of antitumor chemicals in the elimination of tumor cells, their effects on other types of cells in the tumor microenvironment should be investigated. In our study, the effect of OA, which has been shown to have an antitumor effect, on macrophage cells intertwined with the tumor was investigated.

Transformation of macrophage cells was examined without the lethal effect of OA on RAW264.7 cells. In the gene expression analysis of IL1 and IL6 cytokines, which have pro-inflammatory effects, OA was found to be an enhancer. In addition, anti-inflammatory cytokines IL10 were found to be significantly reduced in gene expression analysis. This showed that OA affects macrophage cells in a way that creates a pro-inflammatory response. The formation of a pro-inflammatory environment in the tumor microenvironment promotes the elimination of tumor cells by the immune system. Anti-inflammatory macrophages (M2 type) the most common immune cell in the cancer

microenvironment [22]. Therefore, the transformation of M2 type macrophages into pro-inflammatory type under the influence of OA may retard cancer development.

Dendritic cells, monocytes, T lymphocytes, and B lymphocytes can express CD86, commonly referred to as B7-2. CD86 is an 80 kDa T lymphocyte activation antigen [23]. CD86 stimulates T cell proliferation and generates IL2 through interactions with its ligands, CD28 and CTLA4 [24]. Likewise, when the expression analysis of the genes belonging to the surface markers that are determinant in the transformation of macrophage cells was examined, it was determined that CD86 gene expression increased significantly. Primarily expressed on the surface of monocytes and macrophages, CD163 is a highly selective M2-type tumor-associated macrophage marker. In addition to its ability to fend against inflammation, CD163, a member of the tumor-related macrophage family, is crucial for the growth and spread of tumors. Research has demonstrated a strong correlation between CD163 and malignant tumors, including bladder, lung, colorectal, and breast cancer [25]. The degree of CD163 infiltration influences the prognosis, invasion, metastasis, and proliferation of tumors. Expression analysis of surface markers varied in accordance with the transformation of macrophages compared to cytokine gene expressions.

TGFβ may show a different expression profile in the immune response to tumor [26]. In our study, it was found that TGFβ expression increased in a manner that was not statistically significant. This may be due to TGFβ causing different immunological responses in different conditions.

All the data obtained were evaluated together, it was shown that OA affected pro-inflammatory response-related cellular pathways in RAW264.7 cells. In conclusion, it can be said that macrophages in the tumor microenvironment of OA may have an effect that helps tumor elimination.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: The study is a cell culture study that does not require ethics committee approval.

Author contributions

F.S.C. constructed the main idea and hypothesis of the study. F.S.C. and C.E.G. developed the theory and arranged/edited the material and method section. F.S.C. has done the evaluation of the data in the Results section. Discussion section of the article written by F.S.C. and C.E.G. C.E.G. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

Table 1. Primers used in this study

Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')	PCR (bp)
IL1	GATCCCAAACAATACCCAAAGAAG	AGGTGCTGATGTACCAGTTG	118
IL4	TTGAGAGAGATCATCGGCATTT	CTCACTCTCTGTGGTGTTCTTC	111
IL6	CTTCCATCCAGTTGCCTTCT	CTCCGACTTGTGAAGTGGTATAG	134
IL10	CTATGCTGCCTGCTCTTACTG	GGGAAGTGGGTGCAGTTATT	83
TGFβ	CTGAACCAAGGAGACGGAATAC	GGGCTGATCCCGTTGATTT	101
CD86	GGGCTTGGCAATCCTTATCT	CAGCTCACTCAGGCTTATGTT	139
CD163	CAGACTGGTTGGAGGAGAAATC	CAGCTTCCAGAGACAAGTCAA	101
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	307

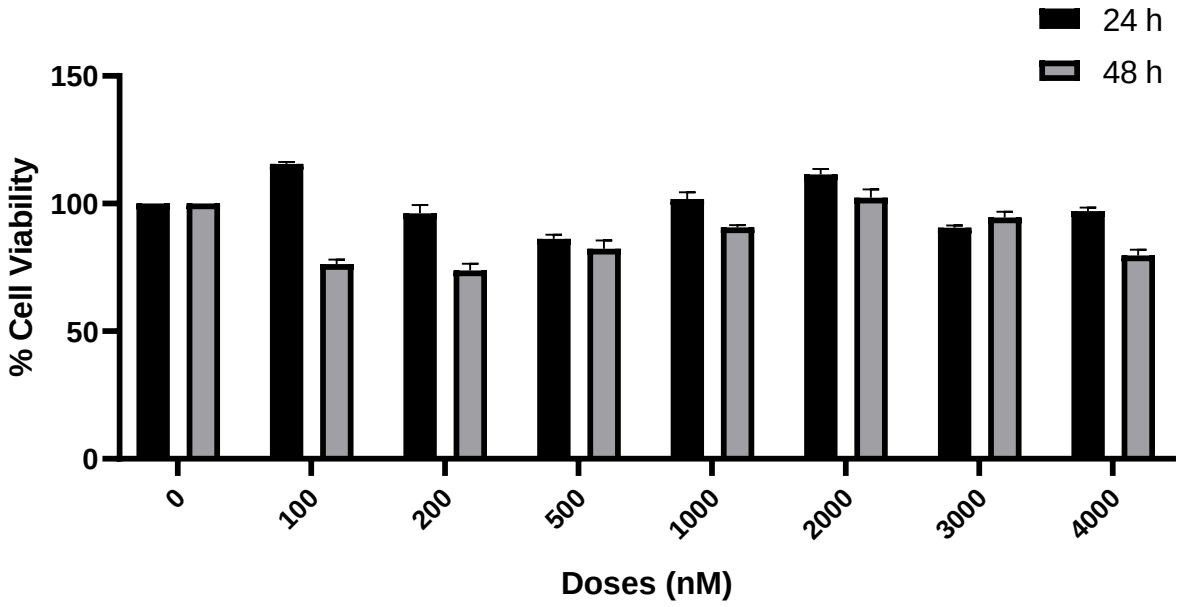

Figure 1. Non-toxic doses range in RAW264.7 cells

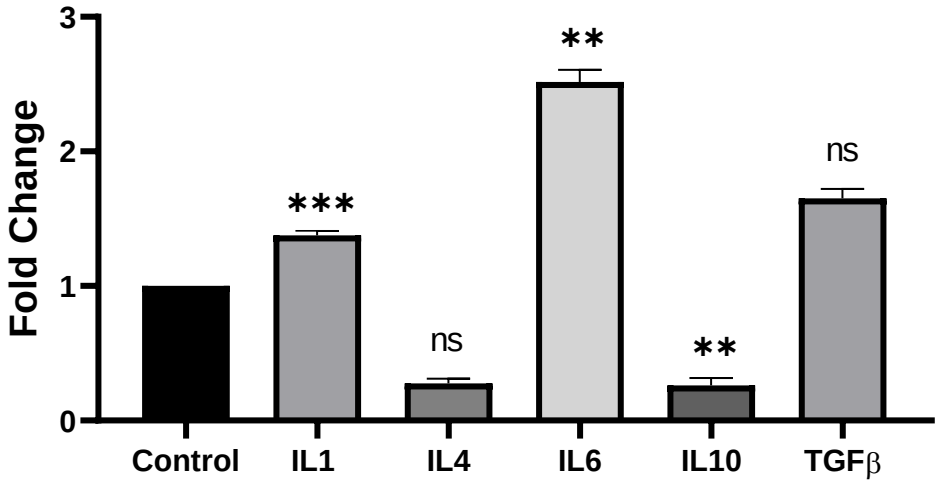

Figure 2. Genes of inflammation expressions

** $p<0.005$, *** $p<0.0005$

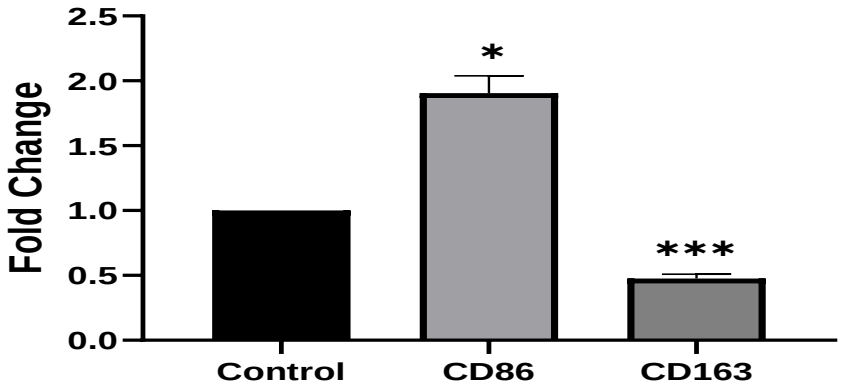

Figure 3. CD86 and CD163 surface markers gene expressions

* $p<0.05$, *** $p<0.0005$

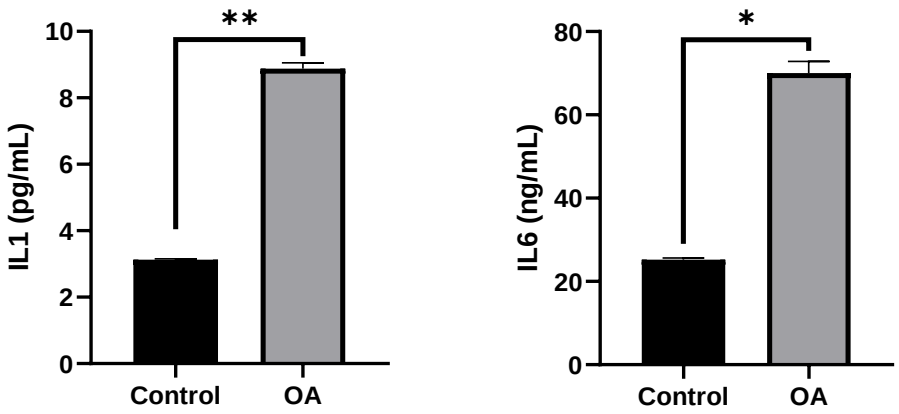

Figure 4. Cytokine amounts expressed by Odoroside A applied cells compared to control

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