İnsan Umblikal Ven Endotel Hücre Kültüründe TNF- lfa İle Oluşturulmuş İn Vitro Greft Versus Host Hastalığı Modelinde Adrenomedullinin Sitoprotektif Etkisi

Effects Of Adrenomedullin On Endothelial Microparticle Release In TNF-Alpha Stimulated In-Vitro Graft Versus Host Disease Model

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Abstract

Background: Allogeneic stem cell transplantation (SCT) is an important treatment modality used in hematological malignant disorders. Graft-versus-host disease (GVHD) is the main complication after allogeneic SCT. It is suggested that endothelial cell damage has an important role in development of GVHD. In this study we investigated the effect of adrenomedullin (AMD) in TNF-α stimulated in-vitro GVHD model since AMD has a protective effect on cells via the regulation inflammatory pathway.

Materials and Methods: Umbilical cord derived endothelial cells were incubated with TNF-α for 2 hours in group1 and with TNF-α+AMD in group2 in order to make in-vitro GVHD model and see the effect of AMD. Microparticles (CD62, CD146, CD31, CD51/61) originated from endothelial cells that are the markers of injury or apoptosis were measured.

Results: We found no significant difference between the microparticle levels in both groups. Although our hypothesis was to find decrement in microparticle levels in gruop2 there were increases and decreases in microparticle levels in both groups ($p>0,05$).

Conclusion: To detect whether there is a protective effect of AMD on development of in-vitro acute GVHD model extensive studies with multiple measurements are needed.

Key words: Adrenomedullin, graft versus host disease, microparticle

Özet

Giriş: Allojeneik kök hücre nakli (KHN) özellikle hematolojik malignitelerin tedavisinde önemli bir yer tutmaktadır. Greft versus host hastalığı (GVHH) allojeneik KHN'nin en sık görülen komplikasyonlarından biridir. Nakil sonrası GVHH'da temel patolojik mekanizmalardan birinin inflamatuar yolak üzerinden endotel hücre hasarı olduğu düşünülmektedir. İnflamatuar yolak üzerinden koruyucu etkisi olan adrenomedullin (AMD)'nin TNF-α ile oluşturulmuş in-vitro GVHH modelindeki sitoprotektif etkisi araştırıldı.

Methods: İnsan umblikal veninden elde edilen endotel hücreleri TNF-α (grup1) ve TNF-α+AMD (grup2) ile iki saat inkübe edildi ve endotel hücre kaynaklı mikropartiküller (CD62, CD146, CD31, CD51/61) ve apoptozis belirteci olan Anneksin-V ölçüldü.

Bulgular: Her iki grupta endotelyal mikropartikül seviyeleri arasında fark yoktu. AMD sonrası bazı ölçümlerde artış tespit edilirken bazı ölçümlerde düşüş tespit edildi (p>0,05).

Sonuç: In vitro akut GVHH modelinde AMD'in koruyucu etkisi olup olmadığını tespit etmek için çok ölçümlü ve büyük çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Adrenomedullin, greft versus host hastalığı, mikropartikül

Introduction

Allogeneic stem cell transplantation (SCT) is an intensive therapy used to treat high-risk hematological malignant disorders and other life-threatening hematological and genetic diseases. The main complication after allogeneic SCT is graft-versus-host disease (GVHD). There is a host tissue damage in GVHD and secreted proinflammatory cytokines, [such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1)] play a role in the development of GVHD (1). It is suggested that endothelial cell damage also has an important role in GVHD after allogeneic SCT (2-5). TNF-α is one of the major mediators that stimulates microparticle (MP) secretion from endothelial cells (6,7). In a recent study it was shown that endothelial MPs increased in patients with acute GVHD and this increment in endothelial MP levels suggested that GVHD may be associated with severe endothelial cell injury (2).

Adrenomedullin (AMD) is an adrenal medulla derived protein and acts as a regulatory protein in the feed-back mechanism between pro-inflammatory and anti-inflammatory cytokines in inflammatory response and also has a cytoprotective effect by inhibiting over production of proinflammatory cytokines like TNF-α, IL-1β (8-11).

In this study we aimed to investigate the cytoprotective effect of AMD on the endothelial cells that were stimulated with TNF-α. We investigated the MP levels after administration of TNF-α and TNF-α+AMD on endothelial cells.

Material and Method

This study was conducted at Hakan Çetinsaya Clinical Research Center Cell Culture Labaratory, Erciyes University School of Medicine. Prior to subject recruitment, the study protocol was reviewed and approved by the university ethics committee, in accordance with the ethical principles for human investigations, as outlined by the Second Declaration of Helsinki and written informed consents were obtained from all the patients. Two umbilical cords of two pregnant women were taken after birth.

Preparation of cells and cell culture was done with a modified procedure conducted by Jaffe et al. (12). Endothelial cells were obtained from human umbilical cord veins. A sterile technique was utilized in all manipulations of the cord. The cord was severed from the placenta soon after birth, placed in a sterile container filled with HANK'S solution, and held at 4°C until processing. Storage time averaged about 30 minutes, and cords were discarded if held more than 2 h. The cord was inspected, and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt 8 Fr feeding tube, 3 cm

long, and the feeding catheter was fixed with an umbilical cord tie. The vein was perfused with 50 ml of PBS to wash out the blood and clean the lumen. The other end of the umbilical vein was then cannulated with a blunt, hubless, 8 Fr feeding tube 3 cm long and fixed. 10 ml of 0.2% collagenase (gibco 17101-015 PAA, No: K.21-240) was infused into the umbilical vein lumen, the both ends/edges of polyethylene tubing was clamped and incubated in a petri dish containing HNAK'S solution at 37°C for 15 minutes. After incubation, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30 ml of PBS. The effluent was collected in a sterile 50 ml conical centrifuge tube (2070, Falcon Plastics, Oxnard, Calif.) containing 10 ml of Medium 199 (TC 199) ' with 20% fetal calf serum (FCS). The cells were centrifuged at 1200 rpm for 10 min and supernatant were removed with a sterile pipet. Endothelial cell medium was added into the centrifuge tube and after this the suspension was vortexed. After having a homogenous mixture the medium was recentrifuged at a 1200 rpm for 10 min. This procedure was done twice. After these procedures 2 ml endothelial cell medium was added on the cells at bottom of the tube and cell were cultured in TC 199 containing 20% FCS, penicillin (200 U/ml), streptomycin (200,xg/ml), and L-glutamine on petri dishes. Petri dishes were incubated at 37 °C under 5% $C0_2$. 24 hours after incubation endothelial cells were washed after with PBS and endothelial cell medium was added. The cells were fed with cell medium every 48 hours. On the fifth day endothelial cells were washed with PBS and 3 ml tripsin-EDTA was added into each flask and incubated at 37°C for 3-4 minutes. After it was shown with invert microscope that the cells leaved the flask they were collected into a centrifuge tube ECM (PAA Endotel Cell Medium U15-002/Millipore supplement 02-102) was added and suspension was centrifuged at a 1200 rpm for 5 min. The cell suspension at the bottom of the tube were taken and used for analyzes. By this method ten tubes were prepared. Every 2 tubes were prepared from one cell culture medium.

1. 10ng/ml TNF-α +0,5ml cell suspension

2. 10^{-6}M AMD and 10ng/ml TNF-α +0,5ml cell suspension

3. 10ng/ml TNF-α +0,5ml cell suspension

4. 10^{-6}M AMD and 10ng/ml TNF-α +0,5ml cell suspension

5. 10ng/ml TNF-α +0,5ml cell suspension

6. 10^{-6}M AMD and 10ng/ml TNF-α +0,5ml cell suspension

7. 10ng/ml TNF-α +0,5ml cell suspension

8. 10^{-6}M AMD and 10ng/ml TNF-α +0,5ml cell suspension

9. 10ng/ml TNF-α +0,5ml cell suspension (24 hours)

10. 10^{-6}M AMD and 10ng/ml TNF-α +0,5ml cell suspension (24 hours)

To see the effect of AMD and TNF-α on endothelial cells, endothelial cells were incubated with AMD+TNF-α and with only TNF-α for 2 hours. In one analyze incubation period/duration was 24 hours. Dosage of AMD was 10^{-6}M and TNF-α was 10 ng/ml. After the incubation of endothelial cells with TNF-α and AMD, MP (CD31, CD51/61, CD62 and CD146) levels were analyzed by flowcytometric studies. After obtaining five endothelial cell mediums 0.5 ml endothelial cells were taken from each mediums and by this way/method each medium was divided into two equal amounts. It was followed by incubating the endothelial cells with only AMD or with AMD+TNF-α.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA). *Independent sample T-test* and *Mann-Whitney U* tests were respectively used in normally and non-normally distributed continuous variables between groups. A two-sided p value < 0.05 was considered to be statistically significant.

Results

Five endothelial cell medium was prepared to analyze the MP levels after incubation of the endothelial cells with TNF-α and TNF-α+AMD for two hours. MP levels were measured by using anti CD62, anti CD31, anti CD51/61 and anti CD146 antibodies with flowcytometric method. There were difference in MP levels after incubation with 10ng/ml TNF-α and 10ng/ml TNF-α+10^{-6}M AMD. While MP levels were high in some measurements after incubation with TNF-α MP levels were also high in some measurements after incubation with TNF-α+AMD. It was seen that Annexin-V positive MP levels related with apoptosis were higher with 24 hours incubation than incubation with 2 hours. There were no difference in MP levels between the groups after incubation with TNF-α and TNF-α+AMD for two hours. Results were shown in graphics.

Discussion

To our knowledge this is the first in the literature. It was not shown that AMD has a protective role against TNF-α stimulated MP increase. Although cytoprotective effect of AMD is known (8-11) it was not shown against tissue/cell injury in artificial GVHD model.

Endothelial cell injury developed after preparation of the patient to allogeneic SCT with radiotherapy and/or chemotherapy suggested to play an essential role in pathogenesis of acute GVHD (2-4). In a study vascular endothelial cell activation and tissue/cell damage was evaluated and seen that soluble vascular adhesion molecule levels increased this suggested that endothelial cell injury or endothelial cell activity may play a role in development of GVHD (13).

TNF-α and several other cytokines plays an important role in the development of GVHD after allogeneic SCT (1, 5, 14). And TNF-α is one of the major mediators makes endothelial cells MP release. It is seen that endothelial MPs also increase in some autoimmune and systemic diseases (6, 15-18).

AMD plays an anti-inflammatory role by inhibiting the overproduction of TNF-α and IL-1β or by inhibiting the inflammation pathway via TNF-α cytokine cascade, this mechanism may have protective effect in GVHD (19-21). AMD reduces the tissue damage with the same mechanism in animal models of sepsis, arthritis and ischemic bowel (22-26). Dosage may be important in protective effect of AMD; in a study it was shown that AMD has a more protective effect with 10^{-3} M and10^{-5} M dosage than 10^{-8} M and 10^{-10} M dosage (27-29). In our study we used the dosage of 10^{-6} M AMD and reported that there were no difference between EMP levels in both AMD and non-AMD groups.

In our study it was detected that MP levels were high in some measurements after incubation with TNF-α and it was also high in some measurements after incubation with AMD+TNF-α. Cytoprotective effect of AMD may also be related with duration of incubation; in some studies it was reported that AMD has more cytoprotective effect with long incubation period (9, 13, 30). In our study we incubated the cells for two hours and have seen that there were not a standard between MP levels in AMD administrated group and without AMD administration group. Only

in one analyze endothelial cells were incubated with AMD for 24 hours and seen that Annexin-V positive MPs were high in CD62, CD146 and CD51/61 groups after incubation with AMD+TNF-α than incubation with only TNF-α. This may suggest that AMD does not have a protective effect against apoptosis.

Conclusion, as to our knowledge; there is no study revealed about the protective effect of AMD on MP release in allogeneic SCT. It is necessary to make an extensive study consist of multiple analysis to detect whether there is a protective effect of AMD on development of acute GVHD.

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Disclosure of interests

All the authors declare that they have no conflict of interest

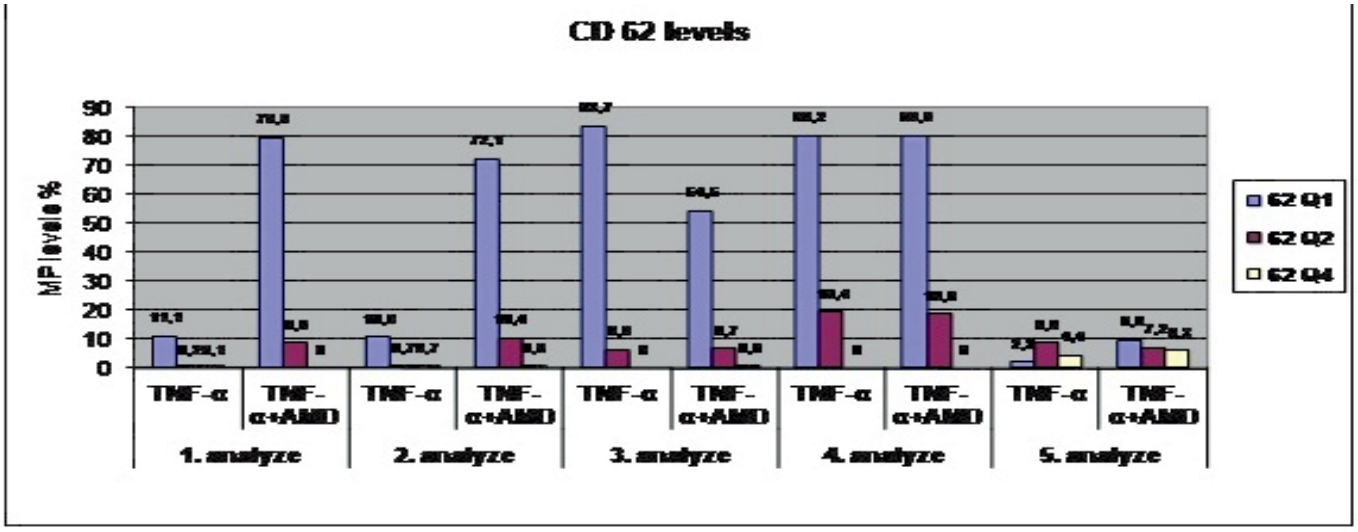

Figure 1: CD62 positive MP levels

p>0,05, Q1 shows the particles positive only for CD62, Q2 shows the particles positive both for Annexin-V and CD62 and Q4 shows the particles positive only for Annexin-V.

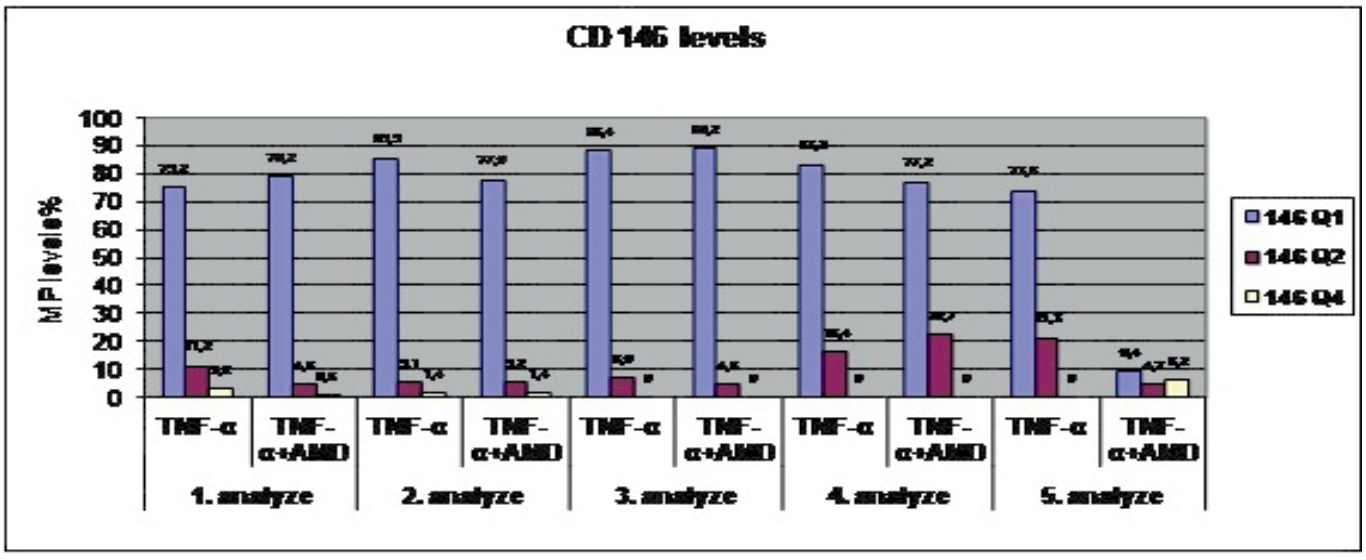

Figure 2: CD 146 positive MP levels

p>0,05, Q1 shows the particles positive only for CD146, Q2 shows the particles positive both for Annexin-V and CD146 and Q4 shows the particles positive only for Annexin-V.

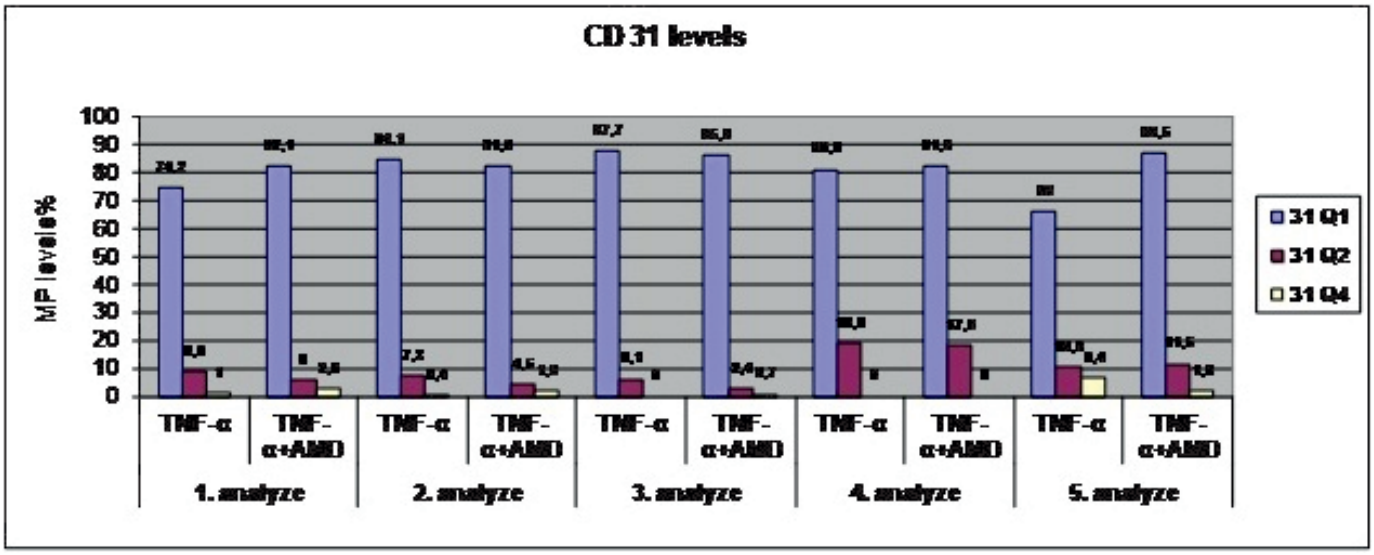

Figure 3: CD31 positive MP levels

p>0,05, Q1 shows the particles positive only for CD31, Q2 shows the particles positive both for Annexin-V and CD31 and Q4 shows the particles positive only for Annexin-V.

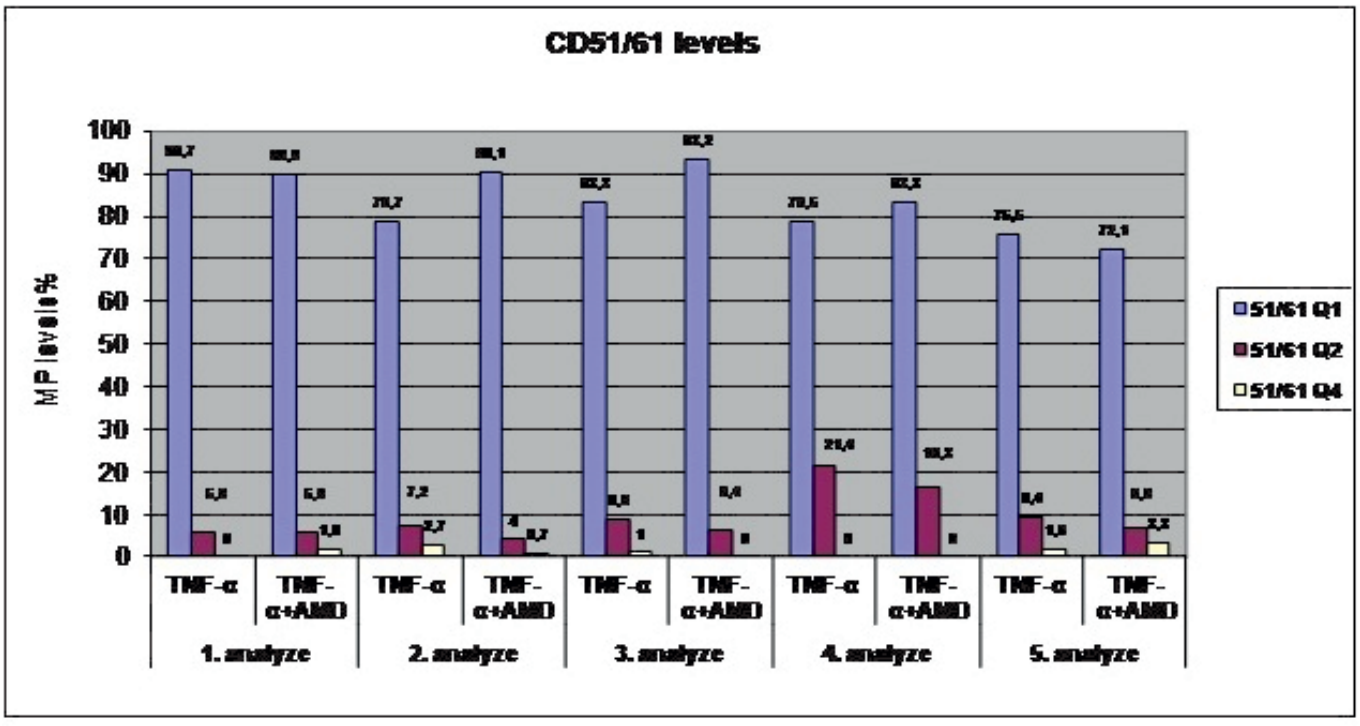

Figure 4: CD51/61 positive MP levels

p>0,05, Q1 shows the particles positive only for CD51/61, Q2 shows the particles positive both for Annexin-V and CD51/61 and Q4 shows the particles positive only for Annexin-V.

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