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The inhibitory influence of adiponectin on the growth of the murine endothelial cell line HECa 10 *in vitro*

Hamujący wpływ adiponektyny na wzrost mysiej linii komórek śródbłonka HECa 10 *in vitro*

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Abstract

Background: Adiponectin, a peptide hormone secreted from the adipose tissue, has anti-diabetic, anti-atherogenic, and anti-inflammatory properties and is also involved in the regulation of angiogenesis. However, there are discrepancies among the results of the published data regarding its pro- or anti-angiogenic properties. The aim of our study was to examine the direct effect of various adiponectin concentrations applied separately or in combination with thalidomide on the growth of the murine endothelial cell line HECa10 in 24- and 72-hour cell cultures.

Material and methods: We used immortalized murine endothelial cell line received from endothelial cells of the mouse peripheral lymph node. The effect of adiponectin was examined at concentrations from 10^{-5} to 10^{-12}M. Thalidomide was used at 10^{-3}M concentration. The growth of HECa10 cells was assessed by the colorimetric Mosmann method.

Results: We found that adiponectin inhibited the growth of HECa 10 line at all examined concentrations in the 24-hour culture, with moderate potency. There were no dose- or time-response effects. In the 72-hour cell culture, adiponectin inhibited the growth with the same or weaker potency and we did not observe its inhibitory effect at 10^{-12}M concentration. There was no beneficial interaction between adiponectin and thalidomide. In this study, however, thalidomide alone did not cause any inhibitory effect on this cell line.

Conclusions: The obtained data show that adiponectin inhibits endothelial cell growth and may participate in angiogenesis regulation as an endogenous antiangiogenic factor. **(Pol J Endocrinol 2009; 60 (3): 166–171)**

Key words: *adiponectin, endothelial growth, in vitro*

Streszczenie

Wstęp: Adiponektyna jest hormonem peptydowym produkowanym przez tkankę tłuszczową wykazującym działanie przeciwcukrzycowe, przeciwmiażdżycowe i przeciwzapalne. Sugerowany jest również jej udział w regulacji procesów angiogenezy. Część autorów uważa, że adiponektyna stymuluje powstawanie nowych naczyń krwionośnych, podczas gdy inni postrzegają ją jako negatywny regulator angiogenezy. Celem niniejszej pracy była ocena bezpośredniego wpływu różnych stężeń adiponektyny zastosowanej osobno lub łącznie z talidomidem na wzrost mysiej linii komórek śródbłonka HECa 10 w hodowli komórkowej 24- i 72-godzinnej.

Materiał i metody: Hodowle prowadzono na mysiej linii komórek śródbłonka HECa 10 otrzymanej w wyniku unieśmiertelnienia pierwotnej hodowli komórek HEC pochodzących z pozakapilarnych naczyń żylnych obwodowych węzłów chłonnych myszy. Wpływ adiponektyny badano w stężeniach od 10^{-5} do 10^{-12}M. Talidomid zastosowano w stężeniu 10^{-3}M. Wzrost komórek śródbłonka oceniano za pomocą metody kolorymetrycznej Mosmanna.

Wyniki: Adiponektyna hamowała wzrost linii HECa 10 we wszystkich badanych stężeniach w hodowli 24-godzinnej. Jej hamujące działanie było umiarkowane. Nie obserwowano zależności dawka-efekt i czas-efekt. W hodowli 72-godzinnej jej hamujące działanie było podobnie nasilone lub słabsze i nie obserwowano jej hamującego działania w stężeniu 10^{-12}M. Nie wykazano korzystnego współdziałania adiponektyny z talidomidem, który sam również nie hamował wzrostu badanej linii w porównaniu z grupą kontrolną.

Wnioski: Wykazany hamujący wpływ adiponektyny na wzrost komórek śródbłonka sugeruje udział tego hormonu w regulacji procesów angiogenezy, jako endogennego czynnika antyangiogennego. **(Endokrynol Pol 2009; 60 (3): 166–171)**

Słowa kluczowe: *adiponektyna, wzrost śródbłonka, in vitro*

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Introduction

Adiponectin, also termed apM1 or GBP28 (and its murine counterpart — Acrp30 or AdipoQ), was originally identified independently by 4 groups of researchers in the years 1995 and 1996 as a protein synthesized exclusively by the adipose tissue [1–4]. In 2005, it was shown that adiponectin is also synthesized and secreted by isolated murine and human cardiomyocytes [5]. Adiponectin has a characteristic NH_2-terminal collagen-like fibrous domain (structurally homologous with collagen VIII and X) and a COOH-terminal complement C1q-like globular domain [1–4]. Via its collagen domain, adiponectin combines to create 3 major oligomeric forms: a low–molecular weight (LMW) trimer, a middle–molecular weight (MMW) hexamer, and a high–molecular weight (HMW) 12- to 18-mer adiponectin [6, 7]. Adiponectin can exist in human plasma as full-length (fAd) or a smaller, globular fragment (gAd) [8, 9]. Two receptor forms for adiponectin were described: AdipoR1, which is ubiquitously expressed, including the abundant expression in skeletal muscles with a high affinity for gAd and low affinity for fAd, and AdipoR2, which is predominantly expressed in the liver with a high affinity for fAd form [9].

Adiponectin circulates at relatively high (μg/ml) concentrations in the plasma, and although adiponectin is secreted from the adipose tissue, paradoxically its plasma level is decreased in obesity [2, 10]. Adiponectin levels correlate negatively with percentage of body fat, fasting plasma glucose, oral glucose tolerance, apolipoproteins (apos) B and E, total and LDL-cholesterol, and uric acid, and positively correlate with HDL-cholesterol and apo A-1 [11, 12]. Its levels are significantly lower in patients with cardiovascular disease [13] and hypertension [14] and in metabolic syndrome [15]. Adiponectin concentration also seems to be gender–dependent (less in men compared than in women), and this is said to be androgen induced [10, 16, 17].

Adiponectin exhibits insulin-sensitizing effects in tissues involved in glucose and lipid metabolism and leads to a reduction of glucose levels *in vivo* [18]. In addition to this effect, adiponectin has potent antiatherogenic and anti-inflammatory properties such as: suppression of TNFα-induced inflammatory changes in endothelial cells, as well as proliferation of myelomonocytic progenitors. It also has an inhibitory effect on phagocytic activity and TNFα secretion from macrophages [19, 20–22]. Moreover, Baranowska et al. [23] suggest that adiponectin may play a role in the mechanisms contributing to prolonged survival, because in their study plasma adiponectin values in a centenarian women group were higher than in any of the other groups.

Dysregulation of angiogenesis is related to atherosclerosis, diabetes, and hypertension. Vascular endothelial cells play a pivotal role in this process. Recent studies show that adiponectin is also involved in regulation of angiogenesis (both receptor forms for adiponectin, AdipoR1, and AdipoR2 are expressed in endothelial cells [24]), but the data about its influence on endothelial cell apoptosis and angiogenesis are conflicting. Some authors suggest that adiponectin stimulates endothelial cell growth and angiogenesis [25, 26], whereas others perceive it as a negative regulator of angiogenesis, which potently inhibits endothelial cell proliferation and migration [27]. Ouchi et al. [25] showed that adiponectin stimulates the differentiation of human umbilical vein endothelium cells (HUVECs) into capillary-like structures and exhibits chemoattractant properties in migration assays. They also tested the effect of adiponectin on blood vessel growth using a mouse Matrigel plug and rabbit corneal assays. Their observations indicate that adiponectin promotes angiogenesis via activation of the 5'-AMP-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3-kinase)-Akt-dependent pathways in endothelial cells. Other studies also provide evidence that due to its ability to stimulate AMPK-dependent signalling, adiponectin plays an important role in the process of ischaemia-induced angiogenesis and stimulates angiogenesis in response to ischaemic stress [28]. Adiponectin has also been thought to be essential for the appropriate development of the retinal vasculature because it stimulates angiogenesis and vascular remodelling in the developing murine retina [29]. The results of the studies of Kobayashi et al. [26] are also consistent with a proangiogenic function for adiponectin. They revealed that the HMW fraction of adiponectin suppresses cell death in serum-deprived HUVEC cultures in a dose-dependent manner. Their data have shown that the AMPK signalling is essential for these anti-apoptotic activities of adiponectin on endothelial cells. In contrast to these data, the study by Bråkenhielm et al. [27] provided evidence that in vitro adiponectin is a direct angiogenesis inhibitor [it inhibits bovine capillary endothelial (BCE) cell growth] and induces apoptosis in activated endothelial cells. Moreover, *in vivo*, in the CAM (chick chorioallantoic membrane) and cornea assays, adiponectin decreases angiogenesis and induces apoptosis in tumours, obtained by implanting T241 fibrosarcoma cells in mice. Other data reported that adiponectin potently suppresses the migration of human coronary artery endothelial cells (HCAEC) induced by VEGF, but does not increase endothelial cell apoptosis [30]. In a mouse model of laser-induced choroidal neovascularization, adiponectin inhibits proliferation of endothelial cells and inhibits vessel growth [31]. Because of these discre-

pancies, the aim of our study was to examine the direct effect of various adiponectin concentrations on the growth of the murine endothelial cell line HECa10. In our study, adiponectin was applied separately or in combination with thalidomide, which is a drug with immunomodulatory and anti-inflammatory properties and, in spite of its past, is currently under evaluation for the treatment of a wide variety of diseases [32]. Thalidomide decreases TNFα production *in vitro* [33] and inhibits IL-6 [34] and IL-12 [35] production by monocytes. In contrast, synthesis of IL-2 [36], IL-4, and IL-5 [37] is enhanced by thalidomide. Thalidomide is thought to have a strong anti-angiogenic activity in VEGF- and FGF-2-induced angiogenesis [38, 39], but some results suggest that this anti-angiogenic effect may only be observed following species-specific metabolic activation of the compound [40].

Material and methods

The murine endothelial cell line HECa10, obtained from the endothelium of mouse peripheral lymph nodes immortalized by transfection using a plasmid construct containing both the gene coding for the large T antigen of simian virus 40 and the Geneticin resistance gene [41], was used in the experiments.

The continuous culture of the cells was maintained in culture flasks (Nunc EasY Flasks 25 cm^2, NUNC). The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 25 mM HEPES buffer (Sigma); 2 g/l sodium bicarbonate (Sigma); 50 μM 2-mercaptoethanol (Sigma); 4 mM L-glutamine (Sigma); 100 U/ml penicillin and 100 μg/ml streptomycin solution (Sigma); 0.1 mM MEM nonessential amino acid solution (Sigma); 1 mM sodium pyruvate (Sigma); 10% heat-inactivated fetal calf serum (FCS, Biochrom) (complete RPMI) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Twice a week, prior to confluency, the cells were harvested after a 2-minute incubation at room temperature in the presence of trypsin-EDTA (0.05 and 0.02%, respectively) in a Hanks-balanced salt solution (Sigma). The cells were collected, washed three times in complete RPMI, centrifuged, and seeded at 5×10^5 cells in 5 ml of fresh medium.

After one of the subsequent trypsinization procedures as described above, the cells were suspended in complete RPMI and 50 μl aliquots of cell suspension (2×10^3 cells for the 24-hour cell culture and appropriately 4×10^3 cells for the 72-hour cell culture) and seeded into 96-well culture plates (Nunclon™ D 96 MicroWell™, NUNC) containing 130 μl of complete RPMI. After 24 hours of preincubation (37°C, 5% CO_2, 95% humidity), the cells were cultured for a further 24 or 72 hours in the presence of the examined substances: adiponectin (previously dissolved in water) at the final concentrations from 10^{-5}M to 10^{-12}M and 10^{-3}M of thalidomide (previously dissolved in DMSO and then in water) applied either alone or jointly. In our study we used human adiponectin [Adiponectin (15–36) (Human); Phoenix Pharmaceuticals, Inc] and thalidomide [(±)–Thalidomide; Sigma]. An equal volume (20 μl) of serum-free culture medium was added to the control wells. The cell growth was assessed by the modified colorimetric Mosmann method, using the EZ4U kit (EZ4U, 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica) following the procedure recommended by the manufacturer. This method is based on the reduction of tetrazolium salt into a coloured soluble formazan product by mitochondrial dehydrogenases in the living cells. The intensity of reaction was estimated via measurement of optical density (OD) using an ELISA reader (λ = 450 nm).

The obtained data are presented as an OD or as the percentage of the control group (% of control); they were statistically analyzed by ANOVA, and the significance of differences between means was determined by LSD (Least Significant Differences). Differences were considered significant if $p < 0.05$.

Results

We found that adiponectin inhibited the growth of the murine endothelial cell line HECa 10 at all examined concentrations in the 24-hour culture. Its inhibitory effect was moderate - between 10.5% (10^{-5}M) and 18.8% (10^{-11}M) of the growth inhibition in comparison to the control group (Fig. 1, Fig. 2). There were no dose- and time-response effects (Fig. 2). In the 72-hour cell culture, adiponectin inhibited the growth only by 10.5% to 14.5% in comparison to the control group. Its inhibitory effect was weaker for the lower concentrations (10^{-10}M and 10^{-11}M) and was not observed at the concentration of 10^{-12}M (Fig. 2, Fig. 3). There was no beneficial interaction between adiponectin and thalidomide at the 10^{-3}M concentration. In this study, however, thalidomide alone did not cause an inhibitory effect on this cell line (Fig. 4).

Discussion

Our data show that adiponectin inhibits endothelial cell growth with a moderate potency, and they are in accordance with the findings obtained in vitro by Bråkenhielm et al. [27] and with data from the *in vivo* study presented by Tytarenko et al. [31]. Bråkenhielm et al. [27] observed that the addition of human adiponectin to BCE cells resulted in a dose-dependent inhibition of FGF-2-stimulated endothelial proliferation. However, in our study there were no dose- and time-response ef-

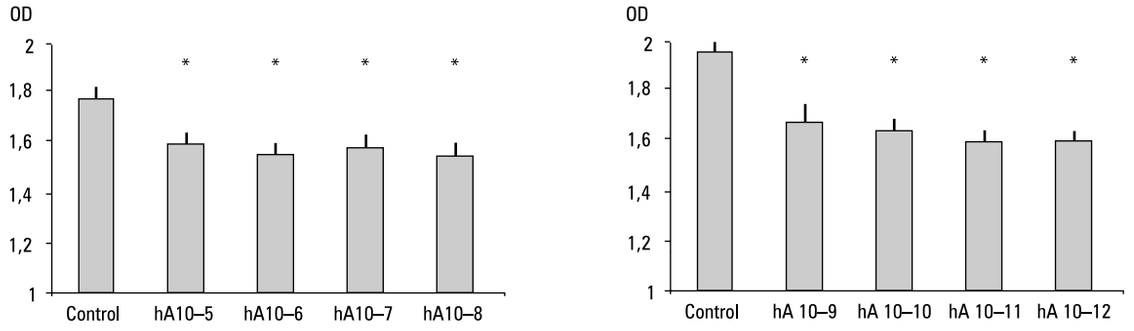

Figure 1. The e*ffect of various concentrations of adiponectin on the growth of the murine endothelial cell line HECa 10 cultured for 24 h; hA 10–5, … hA 10–12: adiponectin at concentrations of 10^{-5}M, …10^{-12}M, OD- optical density; Xe ±SEM; * p< 0.05 vs. control group*

Rycina 1. *Wpływ różnych stężeń adiponektyny na wzrost mysiej linii HECa 10 oceniany po 24 godzinach inkubacji hA 10–5, … hA 10–12: adiponektyna w stężeniu 10^{-5}M, …10^{-12}M, OD — optical density; Xe ± SEM; * p < 0,05* vs. *grupa kontrolna*

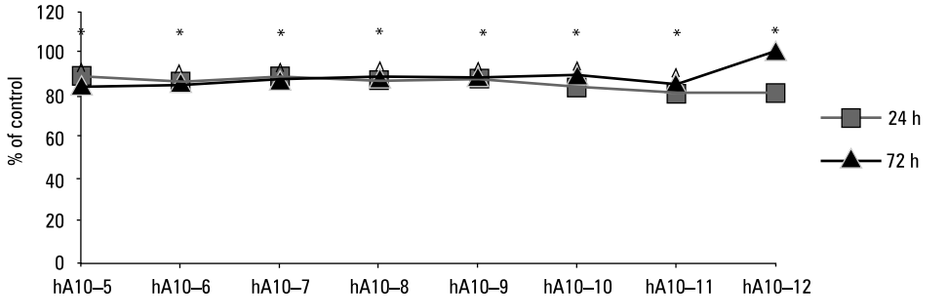

Figure. 2. *The effect of various concentrations of adiponectin on the growth of the murine endothelial cell line HECa 10 cultured for 24 h and 72 h; hA 10–5, … hA 10–12: adiponectin at concentrations of 10^{-5}M, …10^{-12}M*

Rycina 2. *Wpływ różnych stężeń adiponektyny na wzrost mysiej linii HECa 10 oceniany po 24 i 72 godzinach inkubacji hA 10–5, … hA 10–12: adiponektyna w stężeniu 10^{-5}M, …10^{-12}M*

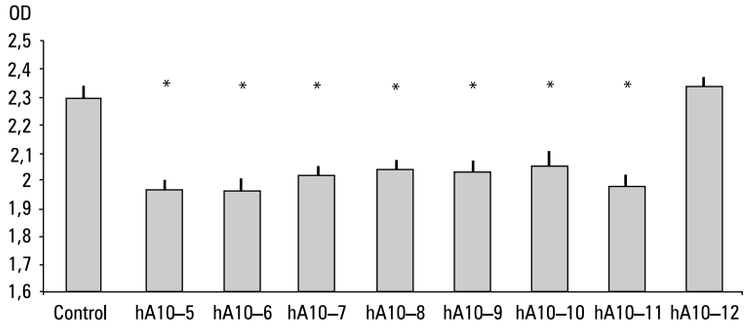

Figure 3. *The effect of various concentrations of adiponectin on the growth of the murine endothelial cell line HECa 10 cultured for 72 h; hA 10–5, … hA 10–12: adiponectin at concentrations of 10^{-5}M, …10^{-12}M, OD — optical density; Xe ± SEM; * p< 0.05* vs. *control group*

Rycina 3. *Wpływ różnych stężeń adiponektyny na wzrost mysiej linii HECa 10 oceniany po 72 godzinach inkubacji hA 10–5, … hA 10–12: adiponektyna w stężeniu 10^{-5}M, …10^{-12}M, OD — optical density; Xe ± SEM; * p< 0,05* vs. *grupa kontrolna*

fects. In *in vitro* studies which used endothelial cells derived from large vessels, such as HUVECs, investigators reported that adiponectin stimulates angiogenesis [25,26]. Bråkenhielm argued that the reason for these discrepancies is the size of the vessels used. Similarly to their study (they used capillary endothelial cells), small vessels were the source of our endothelial cells, so our findings seem to confirm this hypothesis. The molecular form of used adiponectin was different in each study. Some investigators [27] applied a recombinant full-length human adiponectin or mouse adiponectin, others [25] applied recombinant mouse adiponectin

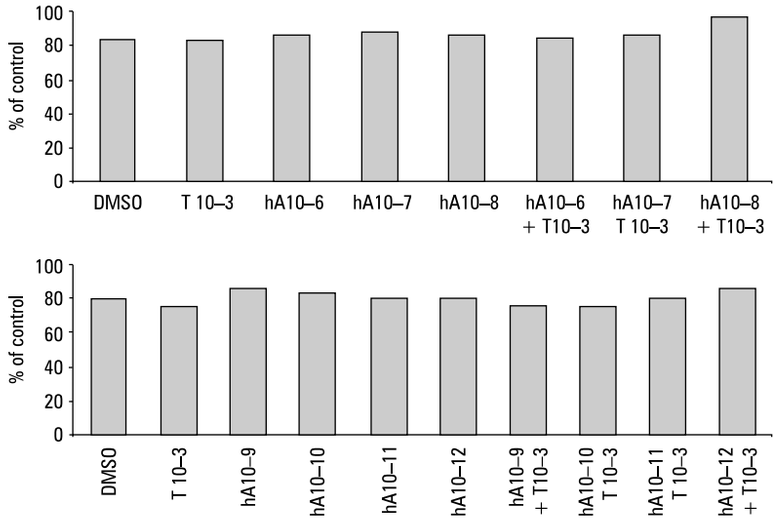

Figure 4. *The effect of various concentrations of adiponectin and thalidomide, applied either alone or jointly, on the growth of the murine endothelial cell line HECa 10 cultured for 24 h; hA 10–5, … hA 10–12: adiponectin at concentrations of: 10^{-5}M, …10^{-12}M, T 10–3: thalidomide at the concentration of 10^{-3}M, DMSO: control for thalidomide*

Rycina 4. *Wpływ różnych stężeń adiponektyny i talidomidu, zastosowanych osobno lub łącznie na wzrost mysiej linii HECa 10 oceniany po 24 godzinach inkubacji hA 10–5, … hA 10–12: adiponektyna w stężeniu 10^{-5}M, …10^{-12}M, T 10–3: talidomid w stężeniu 10^{-3}M, DMSO: kontrola dla talidomidu*

(amid acids 15–247), and we used human adiponectin (amid acids 15–36). This peptide, according to the recommendations, should be rehydrated just before use, and refreezing any unused portions is not allowed, so we used adiponectin from one solution for best results and reproducibility. It is well known that adiponectin forms different oligomeric complexes [6, 7]. Kobayashi et al. [26] reported that the HMW fraction of adiponectin suppressed cell death in serum-deprived HUVEC cultures in a dose-dependent manner, but the trimer or hexamer form of adiponectin had little effect on endothelial cell survival under these conditions. It is likely that the different molecular forms of adiponectin used, and their tendency towards polymerization, also have some influence on the study results. It is also possible that there are multiple potential signalling pathways for adiponectin in endothelial cells and that adiponectin can induce a cascade activation of caspase-8, -9, and -3, which leads to cell death [27], as well as stimulates angiogenesis by promoting the cross-talk between the AMP-activated protein kinase and the Akt signalling [25]. In our experimental model, we only investigated whether adiponectin inhibits or stimulates endothelial cell growth. Moreover, it is also possible that adiponectin inhibits endothelial cell growth (as was shown in this paper) but promotes endothelial cell migration, stimulates the differentiation of these cells into capillary-like structures, or has an anti-apoptotic effect toward endothelial cells [25], and due to all these activities, finally stimulates angiogenesis. Taking into consideration the fact that the process of angiogenesis occurs as an orderly series of events, the apparently contradictory reports of other researchers may be in accordance with our results.

Conclusions

The obtained data show that adiponectin inhibits endothelial cell growth and may participate in angiogenesis regulation as an endogenous anti-angiogenic factor, at least at the level of growth processes of endothelial cells.

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