Original Paper

Combination of TNF- α , Homocysteine and Adenosine Exacerbated Cytotoxicity in Human Cardiovascular and Cerebrovascular Endothelial Cells

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Key Words

Adenosine • Homocysteine • TNF- α • Apoptosis • Cell death • Endothelial cells

Abstract

Disruption to the vascular homeostasis is detrimental in vascular diseases. This study examined how the combination of homocysteine, adenosine and tumor necrosis factor- α (TNF- α) influenced endothelial cell survival. In cultured human-derived cardiovascular (EA.hy926) and cerebrovascular (HBEC-5i) endothelial cells, cell death events were initiated by TNF- α (0.1–10 ng/mL) only when both homocysteine (0.5 mM) and adenosine (0.5 mM) were present. The accelerated cell death events induced by the combination were triggered through excessive apoptosis. This was evident by membrane phospholipid phosphatidylserine externalisation, cell shrinkage and DNA fragmentation, as well as an increase in the expressions and occurrence of active caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) positive cells. Collectively, homocysteine, adenosine and TNF- α are interrelated in the survival of endothelial cells, and this co-existence should be considered in future drug development for cardiovascular and cerebrovascular diseases.

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Introduction

The vascular endothelium, primarily composed of endothelial cells, acts as a physiological defensive barrier between the bloodstream and inner tissues [1, 2]. Continuous exposure

to a wide range of pathological factors from the bloodstream increases the susceptibility of vasculature damage. Excessive apoptosis, a type of programmed cell death, is known to disrupt cellular homeostasis resulting in conditions such as neurodegenerative diseases, liver and heart failure [3-5]. At the vascular endothelium, excessive apoptosis will decrease its gating function, and therefore increase the infiltration of blood-borne substances to the inner tissues [2]. This has been confirmed by excessive apoptosis observed in atherosclerotic lesion, and at the cerebral microvessels following stroke [6, 7]. Therefore, cell death mechanisms are advertently involved in cardiovascular and cerebrovascular diseases.

Hyperhomocysteinaemia, defined as an accumulation of plasma homocysteine, is considered as a risk factor for vascular diseases [8]. Homocysteine is a thiol-containing amino acid involved in the methionine cycle and is important in regulating cellular methylation [9]. Briefly, the transfer of the methyl group from S-adenosylmethionine by methyltransferases results in S-adenosylhomocysteine formation, which is subsequently hydrolysed to homocysteine and adenosine by S-adenosylhomocysteine hydrolase [9]. Since the hydrolysis of S-adenosylhomocysteine is a reversible reaction, the increased levels of homocysteine and adenosine will favor the accumulation of S-adenosylhomocysteine (which is a potent inhibitor of methyltransferases), leading to cellular hypomethylation [10]. It is known that DNA hypomethylation is associated with gene reactivation, and has an impact on the maintenance of cellular homeostasis by dysregulating cell survival [11].

Tumor necrosis factor- α (TNF- α) is a pleiotropic proinflammatory cytokine involved in innate immunity, however, elevated levels are associated with the pathogenesis of inflammatory and vascular diseases [12, 13]. The sensitivity of TNF- α to mediate cell survival or cell death is largely determined by the cell type. TNF- α mediates its regulatory effects on cell survival by ligating the tumor necrosis factor receptor-1 (TNFR1) and tumor necrosis factor receptor-2 (TNFR2), both of which are expressed in most cell lines and primary tissues [14, 15]. It is known that the cytotoxic effects of TNF- α can be mediated through TNFR1 by dissociating the death domain, activating caspase-8 and initiating apoptosis through caspase-3 activation [13, 16]. In contrast, the activation of TNFR2 (which lacks the death domain) can initiate anti-necrotic and anti-apoptotic pathways [13]. Therefore, TNF- α can be both protective and detrimental to cell survival, and is dependent upon the quantitative contribution of TNFR1 and TNFR2 in the cell types.

It has been demonstrated by several groups that homocysteine and adenosine together induced cellular hypomethylation and enhanced the cytotoxic activities of TNF- α in various types of cancer cell, especially those resistant to TNF- α [17-19]. This result could provide avenues for new anticancer treatments. In hindsight, hyperhomocysteinaemia and elevation in TNF- α are considered risk factors for vascular diseases. However, it is still not known whether homocysteine, adenosine and TNF- α will act together to cause endothelial dysfunction. Therefore, the objective of this study was to examine how the combination of homocysteine, adenosine and TNF- α affected endothelial cell survival.

Materials and Methods

Materials

Adenosine was purchased from Merck (Australia). Homocysteine (DL-isoform) was purchased from Santa Cruz biotechnology (USA). Human TNF- α recombinant protein was purchased from e-Bioscience (USA). GelGreen™ was purchased from Biotium (USA). Fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit, phycoerythrin (PE) active caspase-3 apoptosis kit and FITC mouse anti-cleaved PARP (Asp214) were purchased from BD Pharmingen (Australia). Cell culture reagents were from Invitrogen (Australia) and all other chemicals were from Sigma-Aldrich (Australia) unless otherwise stated.

Cell culture and treatments

Human cardiovascular endothelial cell (EA.hy926) and human cerebrovascular endothelial cells (HBEC-5i) were kindly provided by Dr Shanhong Ling (Monash University Central Clinical School, Australia) and Prof Georges Grau (University of Sydney, Australia), respectively. Both cell lines were cultured in 5% CO₂ at 37°C in DMEM/Ham's F12 containing 15 mM HEPES and L-glutamine, and 100 U/ml of penicillin and streptomycin (Gibco BRL, Australia) as previously described [20, 21]. For all experiments, the cells were seeded at a density of 1.0×10^5 cells/mL. Following seeding, 24 - 48 hours were allowed for cells to adhere and become confluent. Once confluent, the endothelial cells were exposed to homocysteine, adenosine and/or TNF- α for 20 hours.

Measurement of cell viability

Three different assays were employed to measure different parameters representing cell viability. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay which measured intracellular metabolism to reflect cell viability was performed as previously described [22]. After the treatment period, MTT (final concentration 0.5 mg/mL) was added and incubated for 4 hours at 37°C. Dimethyl sulfoxide was then added to dissolve the insoluble formazan crystal. The absorbance was measured at 550 nm.

Neutral red uptake assay, sensitive to the intracellular pH gradient, was carried out as previously described [23, 24]. After the treatment period, the medium was replaced with the neutral red working solution which was composed of neutral red in complete medium (50 μ g/mL) for 3 hours. Glacial acetic acid (1%) in ethanol (49%) for destaining. The absorbance was measured at 550 nm.

The experimental procedure for sulforhodamine B assay which measured intracellular protein content was carried out as previously described [25]. After the treatment period, the cells were fixed with 10% (w/w) trichloroacetic acid on ice for at least 1 hour and washed thoroughly with water. After washing and drying, 0.057% (w/v) sulforhodamine B solution was added to each well at room temperature and washed after 30 minutes thoroughly with 1% (w/v) acetic acid to remove the unbound dye. 10 mM Tris base solution (pH 10.5) was added to each well to solubilise the protein bound dye. The absorbance was then measured at 515 nm.

DNA fragmentation by agarose gel electrophoresis

The presence of DNA fragments or laddering pattern is a hallmark indication for apoptosis. DNA fragments extraction was carried out as previously described with minor modification [26, 27]. Briefly, both supernatant and adherent monolayers were harvested by centrifugation at 500 x g. The pellet was then treated with lysis buffer (10 mM Tris, 20 mM EDTA, 1% TritonX-100, pH 7.4) for 30 minutes on ice. The solution was centrifuged at 1600 x g for 5 minutes. The supernatant was then treated with SDS (final concentration 1% (v/v)) and RNase A (final concentration 50 μ g/mL) for 2 hours at 56°C, followed by overnight digestion with proteinase K at 37°C. The DNA-containing solution was further purified with standard phenol/chloroform/isoamyl alcohol procedures followed by ethanol precipitation [26, 27]. DNA fragmentation was assessed on a 2% agarose gel prestained with GelGreen™ (Biotium, USA) at a voltage of 4 V/cm, and the image was captured using GelDoc™ EZ documentation system (Biorad, Australia).

Flow cytometric analyses for apoptosis detection

Apoptosis was detected by flow cytometric analyses using a commercial FITC Annexin V apoptosis detection kit. Briefly, both detached cells and the attached monolayer were collected by centrifugation at 500 x g for 5 minutes. Cells were then resuspended in Annexin-V binding buffer and incubated with FITC-Annexin-V and propidium iodide (PI) for 15 minutes, and 10,000 cells were analysed within 1 hour with FACSCalibur (BD Biosciences, Australia). The data was then analysed with Weasel software version 3.0.2 (Walter and Eliza Hall Institute, Australia). The percentage of apoptotic cells were quantified by combining both early (FITC-Annexin-V⁺/PI⁻) and late (FITC-Annexin-V⁺/PI⁺) apoptotic cells, while negative staining for both FITC-Annexin-V and PI were considered as healthy cells.

Flow cytometric analyses for cell shrinkage

Cell size determined by forward scatter (FSC) was used as a parameter for cell shrinkage as previously described [28]. Briefly, both detached cells and the attached monolayer were collected by centrifugation at 500 x g for 5 minutes. The pellet was then resuspended for flow cytometric analyses. 10,000 cells were analysed using the FACSCalibur and Weasel software version 3.0.2. The results were presented as mean forward scatter.

Fig. 1. Homocysteine and adenosine did not affect the survival of endothelial cells. Cell viability, as determined by MTT dye reduction assay, following treatments with homocysteine (DL-Hcy) alone (A), adenosine (Aden) alone (B), and combination of homocysteine (DL-Hcy) and adenosine (Aden) (C) in both EA.hy926 and HBEC-5i endothelial cells for 20 hours. Cell viability was expressed as a percentage compared to control. All results were expressed as mean $\pm$ S.E.M. from three separate experiments in triplicate.

Flow cytometric analyses for active caspase-3 and cleaved poly(ADP-ribose) polymerase

The cleavage of proteolytic enzyme caspase-3 and poly(ADP-ribose) polymerase (PARP) were determined using PE conjugated anti-active caspase-3 (CPP 32) and FITC conjugated anti-cleaved PARP (Asp 214) antibodies. The experimental procedures were carried out according to the manufacturer's instructions. Briefly, both detached cells and attached monolayer were collected by centrifugation at 500 $\times$ g for 5 minutes. The pellet was then fixed on ice for 20 minutes and washed twice with washing buffer. After fixing, the pellet was resuspended in washing buffer with the antibodies at room temperature for 30 minutes. The pellet was then washed once with washing buffer, and resuspended in appropriate volume of washing buffer for flow cytometric analyses. 10,000 cells were analysed using the FACSCalibur and Weasel software version 3.0.2. The results are presented as mean fluorescence intensity and the percentage of cells with positive staining.

Statistical analyses

Statistical comparisons were performed in GraphPad version 5.02 (USA). The data was analysed by either one way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test or two-way ANOVA followed by Bonferroni's post hoc test where appropriate for multiple comparisons, and Student's t-test for individual comparisons. Data was expressed as mean $\pm$ S.E.M. $P < 0.05$ was considered as statistically significant.

Results

To investigate the cytotoxic response of homocysteine and adenosine, both EA.hy926 and HBEC-5i cells were treated with increasing concentrations of homocysteine or

Fig. 2. TNF- α decreased the survival of endothelial cells only with the presence of DL-homocysteine and adenosine. Cell viability, as determined by MTT dye reduction assay, following treatments with TNF- α with or without the presence of homocysteine (DL-Hcy) and adenosine (Aden) in both EA.hy926 and HBEC-5i endothelial cells for 20 hours. Cell viability was expressed as a percentage compared to control. All results were expressed as mean $\pm$ S.E.M. from three separate experiments in triplicate. *** $P < 0.001$ in comparison with TNF- α alone in the respective cell lines.

adenosine (0.1 - 0.5 mM) for 20 hours and the cell viability was determined using the MTT dye reduction assay. Neither homocysteine nor adenosine treatment showed any significant cytotoxic activities in both cultured endothelial cells up to 0.5 mM concentrations (Fig. 1A and B). Combining homocysteine with adenosine (0.05 - 0.5 mM) also showed no significant cytotoxic effects (Fig. 1C). However, when TNF- α (0.1 - 10 ng/mL) was incorporated with homocysteine (0.5 mM) and adenosine (0.5 mM), the cell death events were significantly exacerbated ($P < 0.001$), while no significant cytotoxic effects were observed when treated with TNF- α alone (Fig. 2).

Additional experiments using different combinations of homocysteine (0.5 mM), adenosine (0.5 mM) and TNF- α (0.5 ng/mL) were performed to ascertain their importance in accelerating endothelial cell death. As measured by the MTT dye reduction assay, significant ($P < 0.001$) cytotoxicity was only observed when the cells were exposed to homocysteine (0.5 mM), adenosine (0.5 mM) and TNF- α (0.5 ng/mL) simultaneously (Fig. 3A). The results were further confirmed by both neutral red uptake (Fig. 3B) and sulfonrhodamine B (Fig. 3C) assays indicating that the homocysteine-adenosine-TNF- α combination significantly reduced cell survival ($P < 0.001$).

To explain the cell death events induced by homocysteine, adenosine and TNF- α , the association with excessive apoptosis was studied. According to the flow cytometric analyses on Annexin-V and PI double-labelling, the proportion of cells with positive Annexin-V staining (i.e. with membrane phospholipid phosphatidylserine externalisation) were significantly increased after treating both EA.hy926 (from $15.75 \pm 1.45\%$ to $64.04 \pm 1.73\%$, $P < 0.001$) and HBEC-5i (from $24.63 \pm 0.60\%$ to $49.21 \pm 1.57\%$, $P < 0.001$) cells with the homocysteine-adenosine-TNF- α combination (Fig. 4A-B). As illustrated in Fig. 4C, the combination also induced cell shrinkage in both endothelial cells with significant reduction in mean forward scatter ($P < 0.001$). Using agarose gel electrophoresis, DNA laddering patterns were detected after homocysteine, adenosine and TNF- α treatment in both cultured endothelial cells indicating the occurrence of DNA fragmentation as a hallmark for apoptosis (Fig. 5).

Two important apoptotic protein markers (e.g. active caspase-3 and cleaved PARP) were also evaluated. The expressions of both active caspase-3 and cleaved PARP were significantly upregulated in both EA.hy926 and HBEC-5i cells following homocysteine (0.5 mM), adenosine (0.5 mM) and TNF- α (0.5 ng/mL) treatment for 20 hours ($P < 0.001$) (Fig. 6A and 6B). In both EA.hy926 and HBEC-5i cells, the occurrence of cells with elevated levels

Fig. 3. Homocysteine, adenosine and TNF- α combination was crucial to accelerate cell death in endothelial cells. Cell viability, as determined by MTT dye reduction assay (A), neutral red uptake assay (B) and sulforhodamine B assay (C), following treatments with different combinations of homocysteine (DL-Hcy), adenosine (Aden) and TNF- α in both EA.hy926 and HBEC-5i endothelial cells for 20 hours. Cell viability was expressed as a percentage compared to control. All results were expressed as mean $\pm$ S.E.M. from three separate experiments in triplicate. *** $P < 0.001$ in comparison with other combinations in the respective cell lines.

of active caspase-3 were significantly increased from $2.68 \pm 0.15\%$ to $33.16 \pm 1.88\%$ ($P < 0.001$), and from $12.14 \pm 0.49\%$ to $42.15 \pm 0.71\%$ ($P < 0.001$), respectively (Fig. 6C) after homocysteine-adenosine-TNF- α treatment. Likewise, the proportion of cells with elevated expressions of cleaved PARP was also significantly increased in both cultured EA.hy926 (from $1.52 \pm 0.11\%$ to $18.65 \pm 0.97\%$, $P < 0.001$) and HBEC-5i cells (from $13.03 \pm 0.39\%$ to $49.74 \pm 0.84\%$, $P < 0.001$) (Fig. 6D).

Discussion

As an interface between blood and inner tissues, the vascular endothelium is readily exposed to a wide range of stimuli that can promote endothelial dysfunction. Some important factors are known to influence cell survival regulation, including TNF- α and cellular hypomethylation, which can be induced by high levels of homocysteine and adenosine [11, 13-15]. Previous studies utilised high concentrations of homocysteine (10 mM) or TNF- α (200 ng/mL) to induce cell death events in primary endothelial cells [29, 30]. This study has achieved lower concentrations in potentiating cell death through combining homocysteine, adenosine and TNF- α in human cardiovascular and cerebrovascular endothelial cells. Our results demonstrated that neither homocysteine (0.5 mM), adenosine (0.5 mM), TNF- α (0.1-10 ng/mL) alone, nor combination of any two factors affected the survival rate of endothelial cells. Only the combination with homocysteine, adenosine and TNF- α resulted in significant

Fig. 4. Homocysteine, adenosine and TNF- α combination induced apoptotic events. Both EA.hy926 and HBEC-5i endothelial cells were treated with homocysteine, adenosine and TNF- α (HAT) for 20 hours. Flow cytometric analysis with FITC-Annexin-V and PI staining revealed that homocysteine (DL-Hcy), adenosine (Aden) and TNF- α induced apoptotic events in both EA.hy926 and HBEC-5i endothelial cells (A). They were expressed as a percentage of the total events by combining the number of cells with FITC-Annexin-V positive staining (B). Mean forward scatter was employed to indicate that the combination induced cell shrinkage in both endothelial cells (C). All results were expressed as mean $\pm$ S.E.M. from six replicas. *** $P < 0.001$ in comparison with blank in the respective cell lines.

Fig. 5. Homocysteine, adenosine and TNF- α combination induced DNA fragmentation. DNA fragmentation, an important marker for apoptosis, was observed in both EA.hy926 and HBEC-5i endothelial cells after treating with homocysteine, adenosine and TNF- α (HAT) for 20 hours.

cell death. The results have been confirmed by three different cell viability assays including MTT dye reduction, neutral red uptake and sulfonrhodamine B assay. Each measures different parameters representing cell survival: MTT dye reduction assay measures intracellular metabolism, neutral red is a vital stain that its uptake is sensitive to cellular pH gradient, and sulfonrhodamine B assay measures cellular protein content [23, 25, 31]. The results observed in endothelial cells were consistent with the previous findings in TNF- α resistant, cancer and

Fig. 6. Homocysteine, adenosine and TNF- α combination activated caspase-3 cascade. Both EA.hy926 and HBEC-5i endothelial cells were treated with homocysteine, adenosine and TNF- α for 20 hours. Flow cytometric analysis revealed that homocysteine (DL-Hcy), adenosine (Aden) and TNF- α upregulated the expressions of active caspase-3 (A) and cleaved PARP (B) in both EA.hy926 and HBEC-5i endothelial cells, as well as increased the occurrence of active caspase-3 (C) and cleaved PARP (D) positive cells. All results were expressed as mean $\pm$ S.E.M. from six replicates. *** $P < 0.001$ in comparison with blank in the respective cell lines.

hepatoma cell lines suggesting similar cell death mechanisms should be involved [17-19]. It is noted that elevated levels of homocysteine and adenosine resulted in the accumulation of S-adenosylhomocysteine and was associated with the induction of cellular hypomethylation [10]. In particular, the extent of DNA methylation has an impact on gene regulation and cell survival [11]. Therefore, we speculated that TNF- α -associated cell death pathways can be sensitive, at least to some extent, to cellular methylation in endothelial cells.

Apoptosis is a form of programmed cell death characterised by cell shrinkage, membrane blebbing, chromatin condensation and nucleus fragmentation [32]. Unlike necrosis, apoptosis is tightly regulated by several intracellular biochemical processes, including the activation of caspase cascade, mitochondrial membrane permeabilisation, intranucleosomal DNA fragmentation and surface exposure of phospholipid phosphatidylserine [31]. Our results showed that the homocysteine-adenosine-TNF- α combination induced excessive apoptosis as evident by the externalisation of phosphatidylserine, cell shrinkage and the presence of DNA fragmentation. Previous findings in hepatoma cells also showed that the homocysteine-adenosine-TNF- α combination induced caspase-8 activation suggesting the involvement of apoptosis [19]. We further explored the possible apoptotic mechanisms focusing on caspase-8 downstream cascade. We demonstrated that the homocysteine-adenosine-TNF- α combination significantly increased the proportion of cells with elevated active caspase-3 levels which are known to be initiated through caspase-8 activation. Similarly, cells with elevated levels of cleaved PARP which can be released as a result of the caspase-3 protease activity were also significantly increased. Our results suggest that there is a tendency that cellular hypomethylation makes TNF- α more favorable towards the apoptotic activities of

TNFR1 through the initiation and activation of caspase-8 and caspase-3 (extrinsic apoptotic pathway). However, further studies are required to evaluate the cross-talk between hypomethylation and TNF- α .

In conclusion, the present study demonstrates that the combination of homocysteine-adenosine-TNF- α reduces the survival rate of both cardiovascular and cerebrovascular endothelial cells, at least in part, through promoting excessive apoptosis via activating the caspase-3. Therefore, homocysteine, adenosine and TNF- α are interrelated in the survival of endothelial cells, and this co-existence should be considered in future drug discovery for cardiovascular and cerebrovascular diseases.

Conflict of interest

The authors declare that they have no competing interests.

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