Up-regulation of autophagy by etanercept treatment results in TNF-induced apoptosis reduction in *EA.hy926* endothelial cell line

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Abstract

Objective

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease associated with a high prevalence of atherosclerosis. Endothelial dysfunction has emerged as a potentially valuable prognostic tool in predicting the development of atherosclerosis. Tumour necrosis factor (TNF) is the main cytokine involved in RA pathogenesis, exerting a pro-atherogenic role. TNF-inhibitors are effective treatments in RA, also improving endothelial function. Regarding this, no experimental data are known about the involvement of etanercept. We investigated the contribution of TNF to endothelial dysfunction and the effect of in vitro treatment with etanercept, with a special focus on autophagy and apoptosis pathways.

Methods

Autophagy and apoptosis were evaluated by Western blot and flow cytometry in EA.hy926 endothelial cells treated with TNF alone or in combination with etanercept for 24h.

Results

Blocking autophagy, TNF was able to induce endothelial cell apoptosis. Co-treatment with etanercept reverted this effect, up-regulating the autophagy pathway.

Conclusion

Our results confirm the protective role of etanercept, by restoring autophagy on TNF-induced endothelial damage.

Key words

autophagy, apoptosis, rheumatoid arthritis, etanercept, TNF endothelial cells

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Introduction

Rheumatoid arthritis (RA) is an autoimmune systemic inflammatory disease characterised by an accelerated atherosclerosis (1, 2). Atherosclerosis is a chronic condition in which lipids and fibrous elements are deposited in the wall of large and medium-sized arteries to activate both the innate and adaptive immune systems. Endothelial dysfunction has emerged as a potentially valuable prognostic tool in predicting the development of atherosclerosis (3). Tumour necrosis factor (TNF) is the main macrophage-derived cytokine involved in the pathogenesis of RA and exerts a pro-atherogenic role (4) by inducing activation of endothelial cells promoting transcription of NF-κB related genes (5). A previous *in vitro* study showed how stimulation of cultured human umbilical vein endothelial cells (HUVEC) with TNF treatment induces changes in the gene expression profile. Upon activation, endothelial cells increase transcription of many genes involved in signal transduction, leukocyte adhesion and chemoattraction such as ICAM-1, TNF receptor-associated factor 1, Bcl3, IL8, fractalkine, E-selectin, lymphotoxin and VCAM-1 (6).

TNF has also been reported to trigger interactions between invading monocytes and vascular endothelial cells (ECs), which subsequently induce endothelial apoptosis (7).

The cytokine binding to its receptor results in aggregation of death domain (8), which contains proteins allowing recruitment of TNF receptor 1-associated death domain (TRADD) protein. TRADD is able to bind both TNF receptor 1-associated protein 2 and Fas associated death domain- containing protein, which in turn induces apoptosis signal-regulating kinase 1 and procaspase-8 activation, respectively (9). Consistent with these findings, increasing recent evidence has indicated that TNF also modulates autophagy, resulting in impairment during atherosclerotic plaque development and contributing to ECs dysfunction (10). How TNF modulates ECs autophagy is not well understood and may differ between different cell types. In human atherosclerotic vascular smooth cells, TNF up-regulates the expression of the autophagy genes LC3 and Beclin 1 and this depends on signalling via the Jun kinase (JNK) pathway (11). In our previous study, we demonstrated that TNF carried on by microparticles purified from RA patients was able to modulate autophagy on ECs by activating NF-κB (12).

During early atherosclerotic stages, basal autophagy seems to be atheroprotective, but becomes dysfunctional in advanced atherosclerotic plaques. During injury, EC autophagy may occur to protect cells from being damaged, while the failure or inhibition of autophagy results in EC apoptosis, leading to the break-down of the endothelium integrity to facilitate the local lipid deposition and plaque formation (13). Nevertheless, our understanding of the mechanisms that control the autophagy of ECs is still limited.

The application of anti-TNF biological agents, such as etanercept (ETA), has dramatically improved the outcome of RA patients, including reduced cardiovascular events, but there are no data investigating the effect on ECs during the treatment of ETA in RA (14).

At the light of these considerations, we aim to investigate *in vitro* contribution of TNF on endothelial dysfunction and the effect of ETA treatment in *EA.hy926* EC line, with a special focus on autophagy and apoptosis pathways.

Materials and methods

In vitro culture of EA.hy926 *cells*

The *in vitro* effects of TNF on the endothelium were evaluated using immortalised human umbilical vein endothelial cell line *EA.hy926* at the third passage (Promocell). Cells were cultured in DMEM containing 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine at 37°C under an atmosphere of 5% CO_2 (6), and were treated with TNF at 10 ng/ml (15, 16) for 4 (data not shown) and 24h. Cells were also treated with etanercept at concentrations of 5, 10 (data not shown) and 15 μg/ml, alone or in combination with TNF, in the presence of protease lysosomal inhibitors E64d and Pepstatin A (PepA) (indicated as "ini" in the figures), both at 10 μg/ml (Sigma-

Aldrich), for 2 h before the end of the culture. The protocol of the study was approved by the Ethics Committee of Sapienza University of Rome (protocol no. 109/18).

Analysis of autophagy by Western blot and flow cytometry

For the analysis of autophagy, *EA.hy926* cell samples (untreated and exposed to TNF and/or etanercept) were lysed in lysis buffer. After SDS-PAGE, Western blot was performed using a rabbit anti-human LC3B and rabbit anti-human p62 antibodies (Abs) (Cell Signaling Technology). Peroxidase-conjugate goat anti-rabbit IgG (Bio-Rad) was used as a secondary Ab. To ensure the presence of equal amounts of protein, the membranes were reprobed with anti-β-actin Ab (Sigma-Aldrich). Quantification of protein expression was performed by densitometry analysis of the autoradiograms (GS-700 Imaging Densitometer, BioRad).

The Cyto-ID kit (ENZO Life Sciences) was used for flow cytometry quantification of autophagy, by determining the percentage of autophagosome formation. At the end of experiments, cells were pelleted and then resuspended in DMEM without phenol red, supplemented with 5% FBS, and diluted Cyto-ID solution was added to each sample. After resuspension, the samples were incubated 30 min at 37°C in the dark. After centrifugation (1500 rpm, 5 min), samples were resuspended in assay buffer and transferred into FACS tubes. FACS Calibur (Becton&Dickinson) was employed for the measurement.

Evaluation of EA.hy926 cell apoptosis

After *in vitro* treatment with TNF and/or etanercept, *EA.hy926* cell apoptosis was analysed by flow cytometry, using a FITC-conjugated Annexin V and propidium iodide apoptosis detection kit, according to the manufacturer's instructions (MBL).

Statistical analysis

Normal distribution of variables was assessed using the Kolmogorov-Smirnov test. Statistical analysis was performed using the program GraphPad Prism v. 6. The Mann-Whitney unpaired test or Student's t-test were used to compare quantitative variables in different groups. *p-values* <0.05 were considered significant.

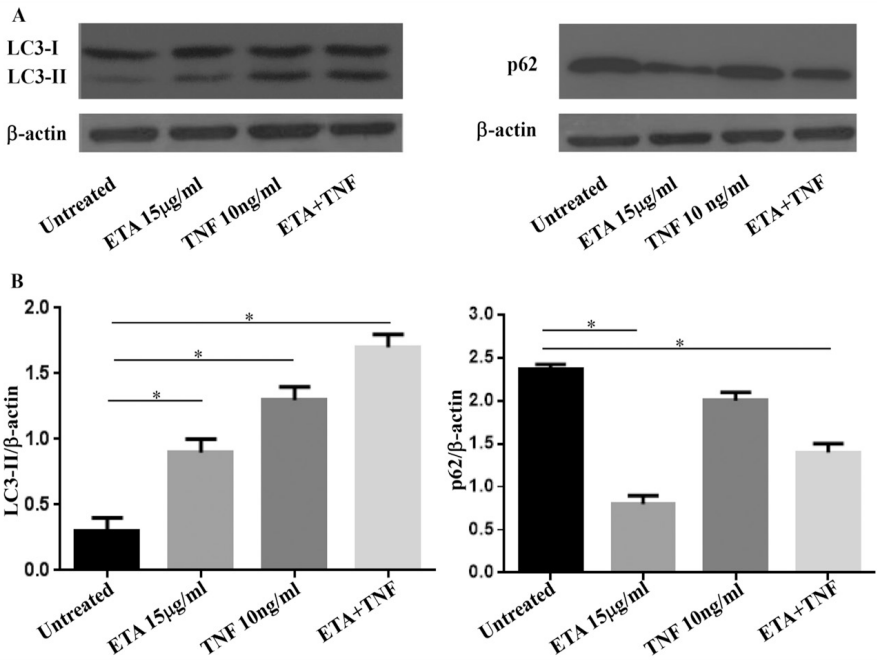

Fig. 1. Modulation of autophagy in *EA.hy926* cells by TNF and etanercept (ETA) treatment.
A: Western blot analysis of LC3-II (left panel) and p62 (right panel) levels in *EA.hy926* cells cultured with TNF and etanercept alone or in combination. The images are representative of 3 independent experiments.
B: Densitometry analysis of LC3-II (left) and p62 (right) levels relative to β-actin is shown. Values are expressed as mean ± SD. *p<0.05

Results

Evaluation of autophagy pathway on EA.hy926 cells treated with TNF and etanercept

Basal levels of autophagy are protective for the endothelium and during injury ECs autophagy may occur to protect the cells from damage.

In our experiments, Western blot analysis showed that both TNF and etanercept (15μg/ml) were able to significantly increase the expression level of LC3-II at 24h in lysates from *EA.hy926* cells. When they were used in combination, the effect on autophagy was more marked. The results of Western blot for p62 protein supported these data, showing an inverse trend, respect to LC3-II (Fig. 1).

In TNF treated cells, LC3-II level was significantly higher than in untreated *EA.hy926* cells, while p62 did not decrease. This result suggested a p62 accumulation due to an autophagy blockade induced by TNF (Fig. 1).

Flow cytometry analysis confirmed that both TNF and ETA were able to significantly increase the percentage of autophagosome formation (Fig. 2). However, in presence of protease inhibitors, only in ETA/ETA+TNF treated cells the percentage of autophagosomes formation was higher, compared to condition without inhibitors (Fig. 2). These results supported the concept that TNF induced a blockade of autophagy, while ETA was able to increase autophagy.

In vitro effect of TNF and etanercept on apoptosis in EA.hy926 cells

TNF is the main cytokine involved in RA pathogenesis and plays a pathogenic role with a direct effect on endothelial cells. Previous clinical studies have shown that TNF inhibitors are able to improve endothelial function. Our *in vitro* results on *EA.hy926* cells showed that TNF significantly increased apoptosis level at 24 h, while etanercept (15μg/ml) alone did not affect this parameter. Interestingly, co-treatment with TNF and etanercept significantly decreased TNF-induced apoptosis (Fig. 3A-B).

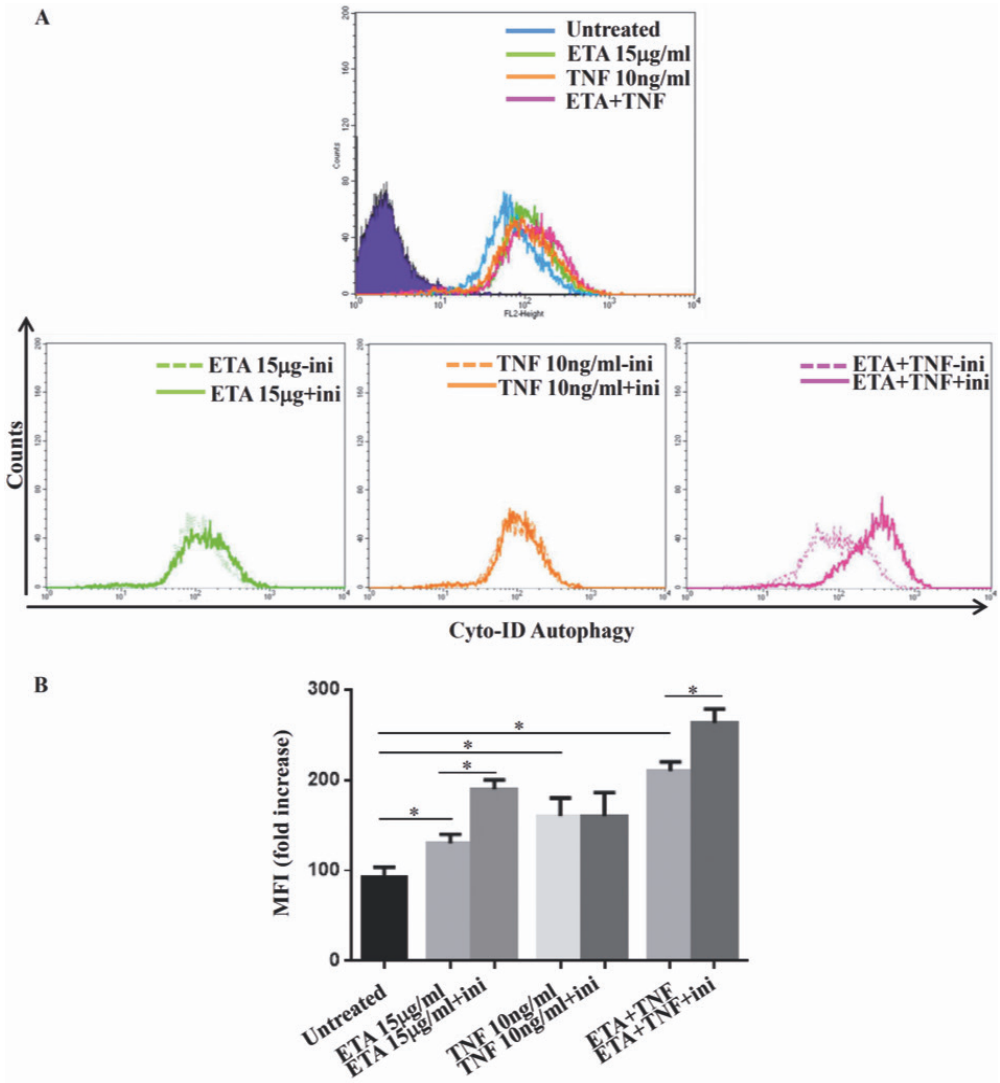

Fig. 2. Flow cytometry analysis of autophagosome formation in *EA.hy926* cells treated with TNF and etanercept (ETA).
A: Flow cytometry staining to detect autophagosome formation, in absence (upper panel) and in presence (lower panels) of protease inhibitors. The results obtained in a representative experiment are shown.
B: Bar graph showing the results obtained from flow cytometry analysis of autophagosome formation. In the ordinate axis, the mean fluorescence intensity (MFI) is reported.
*$p<0.05$.

Discussion

In our study, we investigated TNF contribution to endothelial dysfunction. In particular, we observed that TNF induced *EA.hy926* endothelial apoptosis by a blockade of autophagy, as demonstrated by a p62 accumulation.

In fact, p62 is selectively degraded by autophagy, so the increase in LC3-II shows the buildup of autophagosomes, since p62 was not degraded (17). Hence, our results are indicative of autophagy suppression, according to Opperman and colleagues, who have recently shown as TNF is able to stimulate an accumulation of p62 in rat cardiomyoblasts (18).

Furthermore, we observed the *in vitro* protective effect of etanercept on TNF-induced *EA.hy926* endothelial cell injury. Etanercept was able to reduce apoptosis by an upregulation of autophagy pathway, blocking TNF effect. Indeed, as known in literature and showed by our previous study (19), etanercept can bind TNF, inhibiting its effect on the endothelium.

RA is characterised by an accelerated atherosclerosis. Currently, knowledge regarding the pathogenesis of atherosclerosis in RA and in several other autoimmune diseases includes the chronic inflammation hypothesis, proposed by Ross. In this scenario, the stimulation of EC injury is the initial event (20).

TNF is one of the main cytokines involved in the pathogenesis of the disease and is able to directly compromise endothelial function (21).

Standard therapy of RA includes disease-modifying anti-rheumatic drugs (DMARDs) and biologics, including TNF inhibitors. Very little is known about how biologic agents affect atherosclerosis and endothelial dysfunction, which is directly associated with the development of atherosclerosis and occurs in all stages of the disease.

Previous studies have shown that, in RA patients responder to treatments, TNF inhibitors can improve endothelial function and decrease cardiovascular events by a beneficial effect on vascular wall physiology, increasing the possibility that TNF blockade improves endothelial function and reduces progression of subclinical atherosclerosis (22). Indeed, TNF inhibitors are clinically effective in RA as indicated by the significant decrease of DAS28 (23, 24), and may improve or stabilise vascular morphology and function, including flow-mediated vasodilation (25), common carotid intima-media thickness (26) and arterial pulse-wave velocity (PWV) (27). A recent meta-analysis

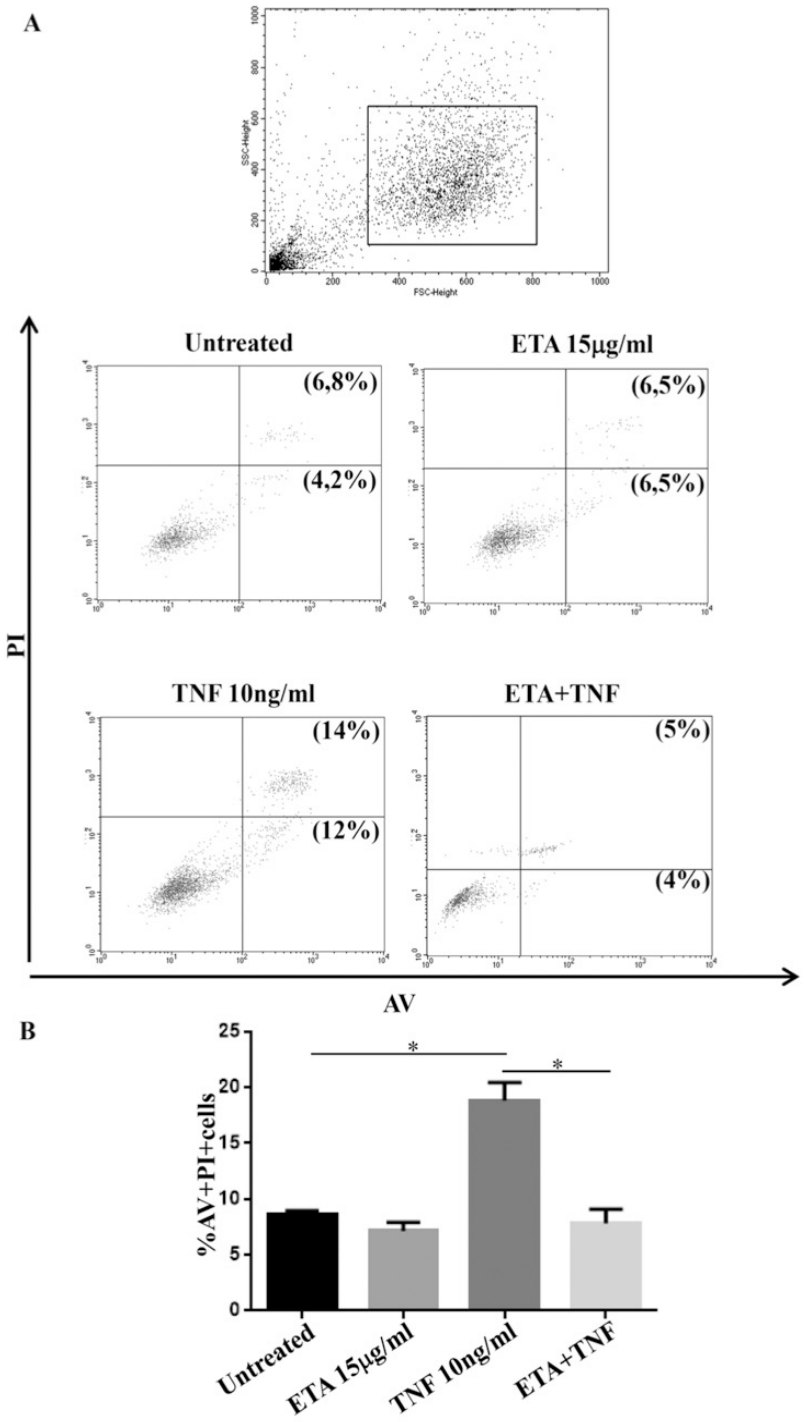

Fig. 3. Effect of TNF and etanercept (ETA) on apoptosis in *EA.hy926* cells.
A: Flow cytometry analysis of apoptosis in *EA.hy926* cells. Results obtained in a representative experiment are shown. Numbers in upper and bottom right quadrants of each plot refer to AV/PI double positive cells and to AV single positive cells, respectively.
B: Mean±SD of the percentages of AV positive cells obtained in 3 independent experiments is reported. **p*<0.05.

focusing on the use of TNF inhibitors showed that anti-TNF treatment induced a significant improvement in aortic PWV and augmentation index and therefore in CV risk (28). In addition, our research group showed a decrease of asymmetric dimethyl arginine, an endogenous inhibitor of nitric oxide synthase that contributes to the impairment of endothelial function, after anti-TNF therapy in RA patients (24).

During injury, EC autophagy can occur to protect the cells from damage, while the failure of autophagy results in EC apoptosis, leading to the breakdown of the integrity of the endothelium (10).

It has been shown that basal autophagy is an essential *in vivo* process mediating proper vascular function (29). Moreover, stress-related stimuli induce autophagy in the arterial wall to protect ECs and smooth muscular cells towards cell death and the initiation of vascular diseases, in particular atherosclerosis. Recently, increasing evidence has indicated that autophagy is impaired during atherosclerotic plaque development and contributes to EC dysfunction (30). During early atherosclerotic stages, basal autophagy seems to be atheroprotective, but becomes dysfunctional in advanced atherosclerotic plaques.

In this regard, our results support a protective role of autophagy on endothelial function. Consequently, the autophagy impairment is responsible for the endothelial damage and the beginning of atherosclerosis. Nevertheless, the mechanisms that control autophagy in endothelial cells are still not well elucidated.

In conclusion, our results suggest a protective role of etanercept in *EAhy.926* ECs, by an up-regulation of autophagy, attenuating the pro-apoptotic effect of TNF, even if studies involving primary endothelial cells would be also needed to better characterise this aspect.

In this view, our results could provide new insights on the protective role of autophagy on the endothelium.

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