

Research Article

Moderate Concentrations of TNF- α Induce BMP-2 Expression in Endothelial Cells

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ABSTRACT

Tumor necrosis factor-alpha (TNF- α) is best-known as a potent pro-inflammatory cytokine involved in many cardiovascular diseases. During vascular calcification, TNF- α has been reported to promote osteogenic differentiation of human vascular smooth muscle cells (VSMC) and mesenchymal stem cells (MSCs). In contrast, there is a lack of data reporting the osteoinductive effect of TNF- α in endothelial cells. In this study, experiments were performed to investigate and determine the optimum dose of TNF- α that induces expression of the osteoinductive factor bone morphogenetic protein (BMP-2) in endothelial cells. Human vein endothelial cells were treated with TNF- α at doses of 0, 2, 5, 10, or 20 ng/mL for 2, 8, or 24 h time intervals. BMP-2 cell expression was evaluated using immunocytochemistry staining by calculating the percentage of BMP-2 positive cells. Apoptosis was determined by counting the number of pyknotic cells. In this study, we found that the optimum dose of TNF- α that induces BMP-2 expression in endothelial cells was 5 ng/mL at the 8 h time interval. Lower (2 ng/mL) or higher (10 and 20 ng/mL) concentrations of TNF- α had minimal effects on BMP-2 expression. Moreover, higher concentrations of TNF- α treatment (10 and 20 ng/ml) at 8 h and 24 h increased the presence of pyknotic endothelial cells, which represent the final stage of apoptosis.

Keywords: TNF- α ; BMP-2; HUVEC; Endothelial cells

INTRODUCTION

TNF- α is best-known as a potent pro-inflammatory cytokine. As a pleiotropic cytokine, TNF- α plays an important role in osteogenesis. Depending on the microenvironment, in ostial tissues such as bone, TNF- α accelerates osteoclast activity leading to bone resorption during the final stages of osteoporosis^{1,2}. In extra-ostial tissues, such as vascular tissue, TNF- α promotes mineral deposition leading to calcification^{3,4}. Calcification leads to decreased vascular elasticity and increased vascular stiffness, which contribute to systolic hypertension^{5,6}. It has been widely reported that TNF- α promotes a shift toward osteogenic differentiation in human vascular smooth muscle cells (VSMCs)⁷. TNF- α plays a pivotal role in regulating the shift of vascular mesenchymal tissues toward an osteogenic phenotype^{8,9}. Moreover, endothelial cells are a primary target of TNF- α -induced organ damage leading to endothelial dysfunction and vascular calcification¹⁰. Some studies have reported that TNF- α -exposed endothelial cells undergo osteogenic differentiation^{11,12}. A shift toward an osteogenic phenotype causes endothelial cells to secrete bone morphogenetic protein 2 (BMP-2), which is a strong bone inductor of osteogenic activity in osteoblasts during bone formation¹³.

The role of TNF- α in VSMCs and mesenchymal stem cells (MSCs) is well documented, however the role of TNF- α in endothelial cells is less defined. It is poorly understood how TNF- α regulates endothelial BMP-2 expression. Hence, the aim of this study was to investigate TNF- α -mediated regulation of BMP-2 expression in a dose- and time-dependent manner.

MATERIALS AND METHODS

This study obtained ethics approval by the Human Ethical Committee of the University of Brawijaya, Malang, Indonesia (141/EC/KEPK/S3/05/2016). Human umbilical vein endothelial cells (HUVECs) were isolated by digestion of umbilical cord veins using 0.05% collagenase (from *Clostridium histolyticum*, Worthington, Lakewood New Jersey, USA) for 15 min. The medium was then changed to remove blood components and non-adherent cells. Adherent cells were then plated on 25 cm² Falcon flasks in RPMI-1640 medium containing 25 mM HEPES and L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10% heat-inactivated fetal calf serum (FCS), and 10% newborn calf serum (NBCS). Cells were then allowed to attach to the dishes for 3 h. When cells reached confluency, ECs were detached with 0.05%

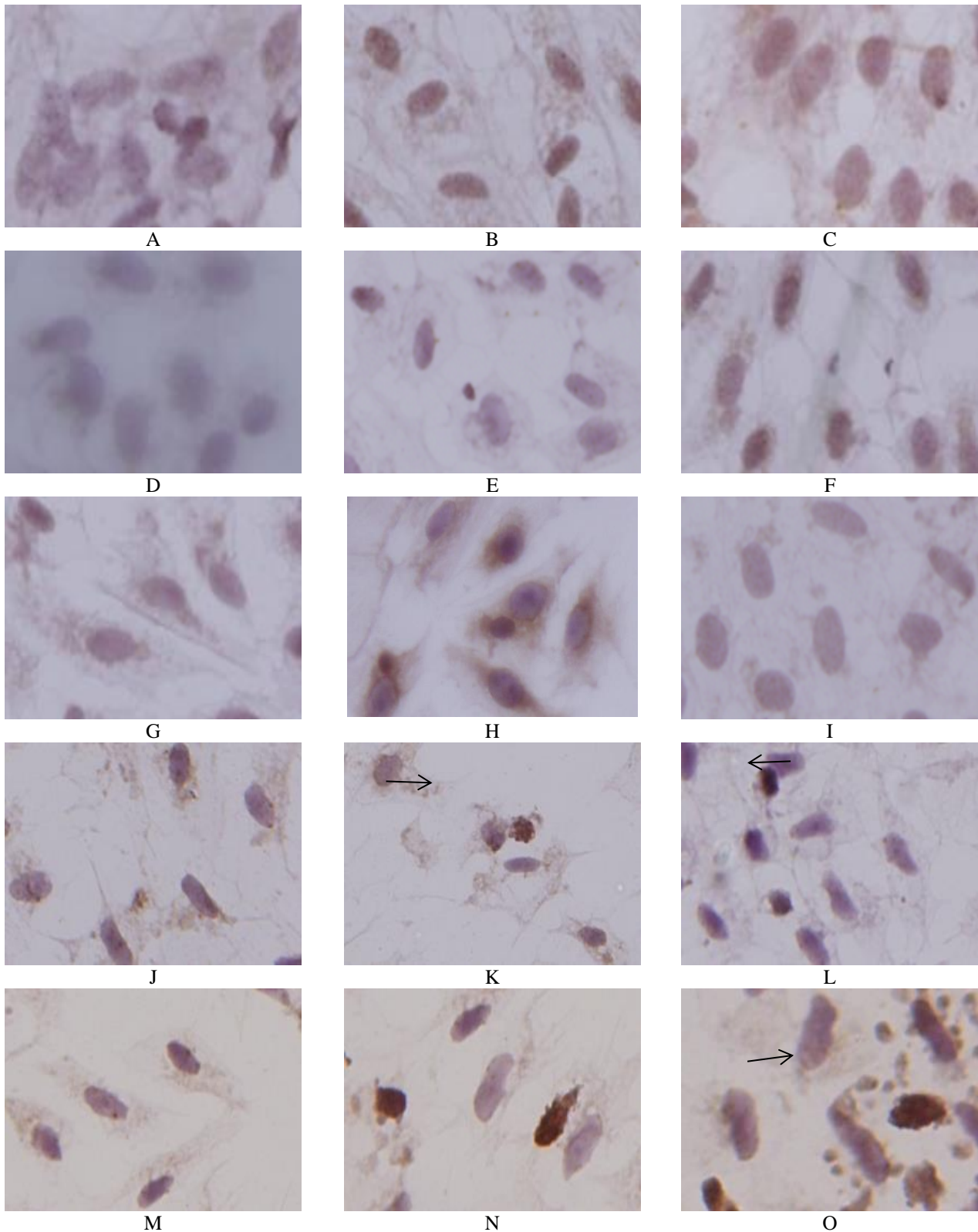


Figure 1: Immunostaining of BMP-2 in control endothelial cultures shows no BMP-2 expression with 0 ng/ml TNF- α at durations of 2 h, 8 h, and 24 h, respectively (A, B, C). At 2 ng/ml concentration of TNF- α , immunostaining shows minimal expression (D, E) of BMP-2 at 24 h (F). Treatment with TNF- α 5 ng/ml (G, H, I) demonstrates optimal expression of BMP-2 immunostaining at 8 h. Furthermore, low expression of BMP-2 is shown along with cellular pyknosis at 10 ng/ml at all times, 2 h, 8 h, or 24 h, respectively (J, K, L) and 20 ng/ml at 2 h, 8 h, or 24 h, respectively (M, N, O) ($P < 0.05$). Arrows point to pyknosis and nuclear karyorrhexis in apoptotic endothelial cells. (M=400x).

trypsin: 0.02% EDTA, and subcultured at a 1 : 3 ratio in the EC cell growth medium above containing, in addition, 15 mg/ml endothelial cell growth supplement (Sigma Chemical Co., UK) and 50 U/ml heparin (Leo Laboratories Limited, UK). Cells were then cultured on 24-well plates (Falcon; BD Biosciences, New Jersey) in M199 medium containing 20% fetal bovine serum, 100 U/ml penicillin-streptomycin, 0.1 mg/ml heparin, and 0.05 mg/ml EGF. Cells were then incubated at 37°C in a 5% CO₂ humidified incubator. At 80% confluency, cells were exposed to increasing doses of TNF-α (0, 2, 5, 10, 20 ng/ml) for 2, 8, or 24 h intervals. Cells were fixed with 5% methanol and stained with BMP-2 antibodies.

Statistical Analysis

Data were analysed by one-way ANOVA and the differences between groups were analysed by LSD post hoc comparison tests. Data are expressed as mean ± standard error of the mean (SEM). P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

In this study, we show that TNF-α increases expression of BMP-2 in endothelial cells in a dose- and time-dependent manner. As shown in Fig 1, the optimal dose of TNF-α that

induces BMP-2 is 5 ng/ml at the 8 h time interval. TNF-α treatment does not increase BMP-2 expression at doses above this concentration because TNF-α at 10 and 20 ng/ml induce cellular apoptosis, particularly at the 8 h time interval, as shown in Fig 3. BMP-2 is not optimally expressed at TNF-α 10 or 20 ng/ml, perhaps because cellular apoptotic signalling is activated before optimum BMP-2 signalling has been reached. As seen in area percentage in Fig 2, BMP-2 expression decreases and cells became more pyknotic, as shown in Fig 3.

In Fig 3, the number of pyknotic cells increased in response to TNF-α at 10 or 20 ng/ml concentrations¹⁴. The induction of pyknosis can be explained due to activation of either the TRAF pathway or the endoplasmic reticulum pathway¹⁵. Low doses of TNF-α at 2 ng/ml results in lower BMP-2 expression at 2 h, 8 h, and 24 h time points. However, different results were obtained by Buendia *et al.* who used 20 ng/ml of TNF-α with 24 h duration to induce endothelial secretion of micro-particles. It is debatable as to whether micro-particle secretion can induce in false positive results due to apoptotic cellular fragments or are a true function of exocytotic cellular vesicles^{16,17}. Endothelial micro-particles are often misunderstood by many researchers as independent membrane vesicular cell

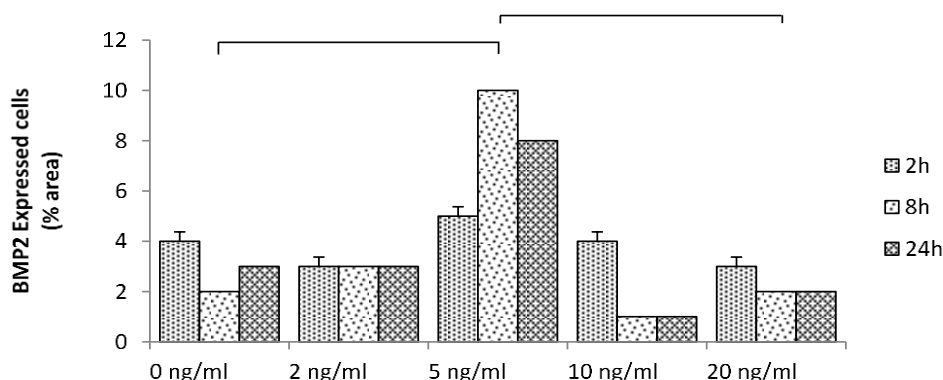


Figure 2: BMP-2 expression optimally increase in TNF-α 5ng/ml at 8h and gradually decrease in higher dose at 10 and 20 ng/ml. In control solution TNF-α 0 and 2 ng/ml shows minimally BMP-2 expression at any time interval 2h, 8h and 24h (* p<0.05).

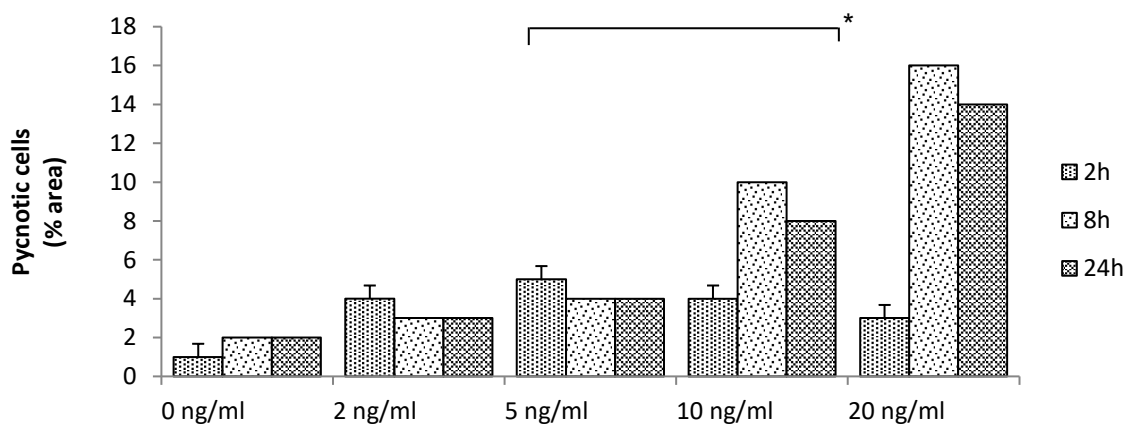


Figure 3: TNF-α 10 and 20 nm/ml at any duration shows more pycnotic cells compare than TNF-α 0, 2 and 5 ng/ml (p<0.05).

products. In addition, many reports have concluded that microparticles are actually apoptotic bodies that are released during the final stages of cellular apoptosis¹⁸.

CONCLUSIONS

The optimum dose of TNF- α that induces BMP-2 expression in endothelial cells without inducing apoptosis is a moderate dose of 5 ng/mL at the 8 h time interval. The lower (2 ng/mL) or higher (10 and 20 ng/mL) concentrations of TNF- α resulted in minimal expression of BMP-2. Moreover, higher concentrations of TNF- α (10 and 20 ng/ml) at 8 h or 24 h time intervals induced apoptosis in endothelial cells. These data may be useful for further studies that examine TNF- α -induced expression of BMP-2 in endothelial cells.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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