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Differential expression of EDF-1 and endothelial nitric oxide synthase by proliferating, quiescent and senescent microvascular endothelial cells

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Abstract

Endothelial Differentiation-related Factor (EDF)-1 is a low molecular weight polypeptide downregulated in endothelial cells exposed to HIV-1-Tat or the phorbol ester TPA. EDF-1 acts in the cytosol as a calmodulin binding protein, and in the nucleus as a transcriptional coactivator. Here, we show that EDF-1 is downregulated in non-proliferating microvascular endothelial cells. Indeed, both quiescence and senescence reduce the levels of EDF-1 and this is due to protein degradation through the proteasome. We also describe a different subcellular localization of EDF-1 which is mainly nuclear in senescent 1G11 cells. Since (i) endothelial nitric oxide (NO) seems to play a role in endothelial proliferation and (ii) NO is an important mediator involved in the control of vascular tone, inflammatory responses and angiogenesis, it is noteworthy that senescence downregulates the expression and the activity of endothelial nitric oxide synthase (eNOS) in microvascular endothelial cells. On the contrary, quiescence does not affect NOS expression and activity. The modulation of EDF-1 in microvascular endothelial cells might offer new insights into the molecular events involved in angiogenesis and in microvascular dysfunctions in the elderly.

Keywords: EDF-1; Endothelial cell; Quiescence; Senescence; Nitric oxide; Angiogenesis

1. Introduction

The endothelium is a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic and immunologic functions. In vivo endothelial cells represent a large population of quiescent cells lining the vessels and microvessels [1]. In certain physiological and pathological conditions, microvascular endothelial quiescent cells become activated and induce angiogenesis, i.e., the formation of new vessels from pre-existing ones. Age-dependent impairment of angiogenesis observed during tumor growth, ischemic vascular disease and wound repair suggests that the phenotypic and functional integrity of the endothelial cells may be compromised in old vs. young animals [2]. Indeed, an emerging theme in tissue repair processes involves the decreased proliferative capacity of aged endothelial cells. Many of the in vivo manifestations of vascular aging are apparent in continuously passaged endothelial cell in vitro [3], underscoring the relevance of using cell culture to examine the contribution of aging to vascular dysfunction. Like normal human somatic cells [4], endothelial cells have a limited capacity to replicate in culture, even under conditions that appear to satisfy their nutritional and mitogen requirements [5].

Endothelial Differentiation-related Factor-1 (EDF-1, also known as hMBF-1) is a novel 16 kDa polypeptide, which may have a role in proliferation since an antisense against EDF-1 inhibits endothelial growth [6]. In the cytosol EDF-1 binds calmodulin (CaM), whereas upon translocation to the nucleus it acts as a transcriptional co-activator [7,8]. EDF-1 function and cellular localization are tightly regulated by

Abbreviations: EDF, Endothelial differentiation-related Factor; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TAF, TATA binding protein-associated factor; PD, population doublings; HUVEC, human umbilical vein endothelial cells; TBP, TATA Binding Protein

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protein kinase C and A [7,8]. EDF-1 has no intrinsic transcriptional activity and does not possess any of the histone modifying activities which have been associated with the modulation of the transcriptional response [9]. Interestingly, MBF-1, the first isolated homolog, is a bridging factor between the steroid orphan receptor fushi tarazu factor (Ftz)-F1 and the basal transcriptional complex [10], suggesting that MBF-1 could recruit the TATA Binding Protein (TBP) at the promoters of Ftz-F1 target genes. In yeast, MBF-1 is a co-activator for the transcription factor GCN4 [11], while in *Drosophila*, it serves as co-activator of the bZIP protein Tracheal Defective/Apontic [12]. The human protein not only binds TBP [7], but it also interacts with the nuclear receptor SF-1/Ad4BP (steroidogenic factor/adrenal 4 binding protein), which regulates steroid hormone synthesis [13]. EDF-1 has also been demonstrated to enhance the transcriptional activity of three nuclear receptors involved in lipid metabolisms, i.e. Liver Receptor Homolog 1, Liver X Receptor alpha and Peroxisome Proliferator Activated Receptor gamma (PPARγ) [9]. Moreover, in myocytes, EDF-1 functions as a transcriptional co-activator of c-*jun* and is necessary for cardiac hypertrophy in vitro [14].

Since (i) EDF-1 binds to different transcription factors and may be considered a TATA binding protein-associated factor (TAF) [9] and (ii) the removal of TAF in mammals inhibits proliferation [15], it is not surprising that EDF-1 plays a role in modulating cell growth.

Because normal human somatic cells have a limited capacity to replicate in culture and progressively reach a state of irreversible growth arrest termed replicative senescence, we evaluated whether a modulation of EDF-1 is detectable in senescent vs. young murine microvascular endothelial cells and whether this correlates with altered cellular functions. To understand whether EDF-1 has a role in endothelial growth, these studies were extended to proliferating vs. quiescent young cells.

2. Materials and methods

2.1. Cell culture

Murine microvascular endothelial 1G11 cells isolated from subcutaneous sponges implanted in mice [16] were a gift from Drs. A. Mantovani and A. Vecchi (Istituto Mario Negri, Milan, Italy). The cells were serially passaged in DMEM containing 10% FCS, ECGF (150 μg/ml) and heparin (5 U/ml) on 2% gelatin coated dishes. Cells were subcultured using 0.05% trypsin, 0.02% EDTA solution. All culture reagents were from Gibco. After contact inhibition cells entered quiescence, whereas sparse cells were actively proliferating, as indicated by tritiated thymidine incorporation [17]. The population doublings (PD) were calculated as $\log_2$ (number of cells at time of subculture/number of cells plated). We defined senescent cells as the culture that do not increase the cell number and remain subconfluent for 2 weeks. We confirmed the senescent phenotype with senescence-associated (SA)-beta galactosidase activity assay as described [18].

2.2. Western blot

1G11 cells were lysed in 10 mM Tris–HCl (pH 7.4) containing 3 mM $MgCl_2$, 10 mM NaCl, 0,1% SDS, 0,1% Triton X-100, 0,5 mM EDTA and protein inhibitors, separated on 15% SDS-PAGE and transferred to nitrocellulose sheets. Western analysis was performed using polyclonal anti-EDF-1 [6], anti-VE cadherin and anti-CD31 (kindly provided by Dr. Elisabetta Dejana), anti-p21, anti-PPARgamma, anti-GAPDH or anti-endothelial nitric oxide synthase (eNOS) antibodies (Santa Cruz–Tebu-bio, sc-6246, sc-7196, sc-25778 and sc-654, respectively). Secondary antibodies were labeled with horseradish peroxidase (Amersham Pharmacia Biotech). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins. The blots were stripped and incubated with an anti-actin antibody (Santa Cruz-Tebu-bio) to show that comparable amounts of protein were loaded per lane. In some experiments, nuclear and cytoplasmic extracts were obtained as previously described [8] and processed by Western blot. All the experiments were repeated at least three times with comparable results.

2.3. Immunofluorescence staining

1G11 cells were seeded on gelatin-coated coverslips, washed, fixed in PBS containing 3% paraformaldehyde and 2% sucrose, permeabilized with HEPES–Triton, incubated with anti-EDF-1 immunopurified IgGs and stained with TRITC-labeled swine immunoglobulins against rabbit [7]. Staining with rabbit non-immune IgGs did not yield any significant signal. Cells were routinely counterstained with FITC-labeled phalloidin (Sigma) to visualize F-actin.

2.4. Determination of proteasome activity

Proliferating, quiescent and senescent cells were lysed in 20 mM Tris–HCl containing 10% glycerol, 5 mM ATP and 0.2% NP-40. After centrifugation, the proteasome activity was determined according to the manufacturer's instructions (20S Proteasome Activity Assay kit, Chemicon). The samples were quantified using a luminescence spectrometer. The proteasome activity was calculated on the standard activity curve of the 20S Proteasome Positive Control. The results are the mean ± standard deviation of two separate experiments in triplicate.

2.5. NOS activity

NOS activity was measured in the conditioned media of 1G11 cells by using the Griess method for nitrate