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Epigenetic regulation of tissue factor inducibility in endothelial cell senescence

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ABSTRACT

Cellular senescence, a programmed state induced by multiple deleterious triggers, is characterised by permanent cell-cycle exit and altered gene expression and cell morphology. In humans it is considered a tumor suppressor mechanism, mediating removal of damaged or mutated cells from the cell-cycle pool, and may also contribute to the ageing process. In this study, we show that senescent human umbilical vein endothelial cells lose their ability to induce tissue factor (TF), a transmembrane protein with important roles in hemostasis and cancer progression, in response to thrombin or – independently of cell-surface receptors – phorbol-12-myristate-13-acetate. This phenomenon could not be explained by senescence-related alterations in the downstream signal transduction cascade or by accelerated TF mRNA degradation. Rather, using chromatin immuno-precipitation we could show that loss of TF gene inducibility during senescence occurs following chromatin remodelling of the TF promoter resulting from hypo-acetylation of histone H3. These findings were reversible after transduction of presenescent cultures with telomerase reverse transcriptase, enabling late-passage cultures to escape senescence. These results extend the involvement of heterochromatic gene silencing in senescence beyond cell cycle-related genes and suggest a novel anti-cancer mechanism of senescence through inhibition of TF inducibility.

1. Introduction

Cellular senescence was originally described as an irreversible growth arrest following serial cell division in culture (Hayflick and Moorhead, 1961). It has since become recognised as a programmed state induced by a variety of deleterious triggers, characterised by permanent cell-cycle exit and accompanied by altered gene expression and cell morphology. In humans it is considered a potent tumor suppressor mechanism, mediating removal of aged, damaged or mutated cells from the cell-cycle pool (Campisi, 2003; Collado et al., 2007).

Access to the vasculature is a prerequisite of tumor growth, invasion and metastasis. The 47 kDa transmembrane protein tissue factor (TF), besides being a primary initiator of the blood coagulation cascade, is prominently involved in these processes (Kasthuri et al., 2009; Garnier et al., 2010; van den Berg Yascha et al., 2012). Gene targeting studies have shown that expression of TF is essential for vascular development (Carmeliet et al., 1996), and TF is markedly upregulated in many cancer cells and their associated endothelial cells (EC) (Rak et al., 2006). Similarly, loss of tumor suppressors promotes the upregulation of TF in concert with oncogenic K-ras, and selective reduction in TF expression in colorectal cancer cells reduced tumor growth in mice (Yu et al., 2005). The involvement of TF at the crossroads of tumor and vasculature is such, that it is considered a promising target for anticancer therapies (Kasthuri et al., 2009; van den Berg Yascha et al., 2012).

Abbreviations: TF, tissue factor; EC, endothelial cells; PKC, protein kinase C; JNK, c-jun terminal NH2-kinase; CPD, cumulative populations doublings; SA-b-gal, senescence-associated beta galatosidase staining; ChIP, chromatin immunoprecipitation; PMA, phorbol-12-myristate-13-acetate; K9H3Ac, acetylated histone H3 on lysine 9; hTERT, telomerase reverse transcriptase.

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We have previously shown that TF expression is tightly regulated in human EC, and its surface activity is virtually absent in the quiescent endothelium (Stähli et al., 2006). This changes dramatically upon stimulation of EC with a number of mediators and cytokines, including thrombin, tumor necrosis factor-α, lipopolysaccharides, and vascular endothelial growth factor (Steffel et al., 2006). Most of these inducers of endothelial TF share similar signal transduction pathways, involving protein kinase C (PKC), and the mitogen-activated protein kinases p38, p44/42 (ERK), and c-jun terminal NH2-kinase (JNK) (Steffel et al., 2006).

In this study we investigated alterations in TF expression and inducibility – and their regulatory mechanisms – occurring with the onset of senescence in normal human endothelial cells.

2. Materials and methods

2.1. Cell culture, induction of tissue factor

First passage cryopreserved Human Umbilical Vein Endothelial Cells (HUVEC, Clonetics) were cultured in endothelial growth medium (EGM, Lonza) according to the manufacturer's instructions and supplemented with 5% FCS. Cells were cultured in 25 cm^2 flasks and serially passaged until they reached senescence as described (Kurz et al., 2000). The number of cumulative population doublings (CPD) was calculated using the formula PD = (ln[number of cells harvested] − ln[number of cells seeded])/ln 2.

For experiments, early passage (young, 10–12 CPD) and senescent (35–40 CPD) cells were grown in parallel in 3 cm culture dishes (unless otherwise indicated). Differences in proliferative rates between young and senescent cells were compensated for by adapting seeding densities ($1.25–7.5 \times 10^3/cm^2$). At confluence, cells were synchronized to G0 phase in EGM containing only 0.5% FCS for 24 h and treated with either 4 U/ml thrombin (#T7009 Sigma) or 10 ng/ml PMA (#524400 Calbiochem).

2.2. SA-β-galactosidase staining

Cells were washed with PBS and fixed in 3% paraformaldehyde (PFA) for 3 min at room temperature. Cells were washed in PBS to remove PFA and incubated in freshly prepared SA-β-gal solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside(X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 40 mM citric acid and pH was adjusted with NaH_2PO_4 to 6.0) at 37 °C for 24 h. After several washings with PBS cells were dehydrated with graded ethanol and observed under a bright-field microscope for presence of SA-β-galactosidase staining. The percentage of SA-β-gal-positive cells was determined by cell counting in each sample (Kurz et al., 2004).

2.3. Western blotting

Cells were washed with ice cold PBS and lysed in lysis buffer (50 mM Tris–7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM NaF, 0.1 mM Na_3VO_4 and 1 mM DTT) for 10 min. Lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C, and protein concentration in the supernatant was determined using the Bradford assay. 40 μg of total protein was used for Western blotting as described (Stähli et al., 2006). Blots were blocked in 5% milk powder and probed with respective primary antibodies (Human TF (#4503, American Diagnostica), PKC H300 (sc-10800, Santa Cruz), PAR-1 (sc-13503 Santa Cruz), CD-31 (BBA7, R&D Systems), ICAM-1 (Zymed Laboratories 075403), phosphorylated forms of p38 (#9216S, Cell Signalling), p44/42-ERK (#9101S, Cell Signalling), JNK (#9255S, Cell Signalling) and total of p38 (#9212, Cell Signalling), ERK (#9102, Cell Signalling), total JNK (#9258, Cell Signalling), IκB-α (#9242, Cell Signalling) and HRP conjugated secondary antibodies. Blots were developed with the ECL Plus (GE Healthcare) detection system. Results were normalised to corresponding loading control proteins.

2.4. TF activity

100,000 cells were growth arrested as described in Section 2.1. Cells were washed three times with PBS, and factor Xa generation was determined using a chromogenic assay according to the manufacturer's instructions (#846, American Diagnostica). Briefly, cells were incubated with 25 μl of factor VIIa and 25 μl of factor X for 15 min at 37 °C on a plate shaker. 25 μl of spectrozyme-FXa was added and incubated at 37 °C for an additional 20 min. Absorbance was read at 405 nm in an ELISA reader (Payeli et al., 2008).

2.5. Induction of stress-induced senescence

HUVECs were incubated with 200 μM H_2O_2 in EGM/5% FCS. After 2 h, the oxidant medium was replaced with fresh EGM/5% FCS, and 48 h later cultures were replated for SA-β-Gal staining and for assessment of TF inducibility, as described in Section 2.2.

2.6. Purification of membrane fraction

HUVECs were stimulated with PMA for 30 min and harvested by trypsinization. Pelleted cells were resuspended in 6.8 ml of homogenization buffer (50 mM Tris pH 7.5, 50 mM Mannitol, 2 mM EGTA, 50 μM PMSF) at 4 °C and homogenized by 5 freeze-thaw cycles followed by douncing. The homogenate was centrifuged at 500 × *g* for 10 min, and the supernatant was collected and centrifuged at 100,000 × *g*. The membrane fraction pellet was resuspended in storage buffer (50 mM sucrose, 100 mM KNO_3, 12.5 mM $Mg(NO_3)_2$, 10 mM Tris pH 7.4) and concentrated using Centricon 30 kDa cut-off tubes (Millipore). Protein concentrations in the membrane fractions were determined by Bradford assay.

2.7. Protein kinase C (PKC) activity assay

PKC activity was quantified using a kinase activity assay kit (EK S-420A, Stressgen Bioreagents) according to the manufacturer's instructions. 30 μg of total protein was added to substrate precoated 96-well plates; the reaction was initiated by addition of ATP and incubated for 30 min at 37 °C. Phosphorylated substrate peptide was detected with phospho-peptide specific antibody and corresponding horse radish peroxidase-conjugated secondary antibody. A 3,3,5,5-tetramethyl benzidine substrate was used to develop the colour and absorbance was read at 450 nm. All experiments were performed 5 times and results are presented as percentages of PKC activity compared to control cells.

2.8. Real time PCR

Total RNA was extracted from HUVEC using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 4 μg of total cellular RNA was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers (GE Healthcare). 2 μl of cDNA was used for real time PCR as described (Stähli et al., 2006). Briefly, SYBR Green JumpStart (Sigma) was used according to the manufacturer's instructions. The following primers were used to amplify full length human TF (F3): sense 5′-TCCCCAGAGTTCACACCTTACC-3′ (bases 508–529 of F3 cDNA; NCBI no. NM001993); antisense 5′-TGACCACAAATACCACAGCTCC-3′ (bases 892–913 of F3 cDNA; NCBI no. NM001993); human ribosomal L28: sense 5′-GCATCTGCAATGGATGGT-3′, antisense 5′-TGTTCTTGCGGATCATGTGT-3′. The amplification programme consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles with a denaturing phase at 95 °C for 30 s, an annealing phase at 60 °C for 1 min, and an elongation phase at 72 °C for 1 min. PCR products were analysed on an ethidium bromide stained 1% agarose gel. In each real-time PCR run for TF and L28, a calibration curve was included to quantify the amplified copies.

2.9. TF promoter activity

TF promoter (−227 bp to +121 bp) was cloned into the vector VQAd5/hTF/Luc containing the luciferase reporter gene and was used for production of an adenoviral vector (Ad5/hTF/Luc) as previously described (Mackman, 1995). HUVEC were transduced at a moi of 50 pfu/cell for 1 h at 37 °C and maintained for another 24 h in EGM. Transduced cells were rendered quiescent for 24 h in medium containing 0.5% FCS and then incubated with 10 ng/ml PMA or carrier for 5 h. Cells were then lysed in Glo lysis buffer (Promega) and luciferase activity was determined in cell lysates using a luminometer (Berthold Technologies). Total protein concentration of the lysates was used for normalisation of luciferase activity.

2.10. TF mRNA stability assay

Two hours after stimulation with 10 ng/ml PMA, transcription was stopped by addition of 10 μg/ml daunorubicin (D8809, Sigma). At the indicated time points following addition of daunorubicin, cells were washed with PBS and lysed with TRIzol reagent for RNA isolation. RNA was processed for real-time PCR analysis as described above. Values were plotted as a function of time, and the RNA half-life was calculated.

2.11. Adenoviral transductions

Late passage, partially senescent HUVECs were transduced with hTERT or LacZ adenoviral vector at moi of 50 pfu/cell for 1 h at 37 °C. Cells were retransduced with the virus every 10–15 day until re-emergence of a non-senescent phenotype. After infection, residual virus was removed and cells were maintained in EGM. Transduction efficiency was quantified in LacZ-transduced cells by β-galactosidase staining.

2.12. Chromatin immuno-precipitation

1.5×10^6 HUVEC were lysed as described for Western blotting and processed for chromatin immuno-precipitation assay (17–375, Upstate) according to the manufacturer's instructions. Briefly, chromatin was digested with streptococcal nuclease to achieve fragments of 150–300 bp. Acetylated chromatin was immunoprecipitated using anti-acetyl K9-Histone3 antibody (Millipore Cat. 17-615) and IgG isotype