FEBS Letters 582 (2008) 886–890

Interleukin-1 receptor antagonist (IL-1ra) modulates endothelial cell proliferation

Rachael M. Dewberry*, Andrea R. King, David C. Crossman, Sheila E. Francis

Cardiovascular Research Unit, School of Medicine and Biomedical Sciences, University of Sheffield, Royal Hallamshire Hospital, Sheffield S10 2RX, United Kingdom

Received 5 December 2007; revised 5 February 2008; accepted 7 February 2008

Available online 20 February 2008

Edited by Masayuki Miyasaka

Abstract **Endothelial cell (EC) lifespan controlled by the IL-1 family of cytokines is an important determinant of susceptibility to artery wall disease. Here we show that EC lacking intracellular interleukin-1 receptor antagonist (IL-1ra) have a reduced lifespan compared to controls. Over expression of IL-1ra enhanced proliferation via cyclin dependent kinase 2 activity and retinoblastoma protein phosphorylation. This was not seen in EC lacking IL-1 receptor 1 (IL-1 signalling ability), nor apparent using other stimuli e.g. TNFα. These data suggest that IL-1ra has a specific and receptor-dependent function to control the growth and lifespan of EC.**

Keywords: Endothelial cell; IL-1ra; Proliferation

1. Introduction

The healthy, unactivated endothelium is a non-thrombogenic single layer of cells that lines the luminal surfaces of blood vessels. Disturbance of endothelial integrity leads to a variety of disease states, underlining the importance of biological and molecular mechanisms concerned with endothelial cell (EC) renewal and preservation.

Chronic inflammation, mediated by the pro-inflammatory cytokine interleukin-1β (IL-1β), has a recognised role in the pathophysiology of cardiovascular disease [1] with IL-1β expression localised to the endothelium of atherosclerotic vessels. ECs undergo a finite number of cell divisions before growth arrest or replicative senescence; modulated in part by IL-1. There is a marked accumulation of IL-1 in aging ECs and antisense oligonucleotides targeted to IL-1α extend EC lifespan [2,3], whilst addition of exogenous IL-1β inhibits the proliferation of cultured ECs [4].

The naturally occurring antagonist, interleukin-1 receptor antagonist (IL-1ra), blocks signalling of IL-1 by binding to the IL-1 type 1 receptor (IL-1R1). There are both secreted and intracellular isoforms of IL-1ra [5], the secreted form bestows protection in animal models of cardiovascular disease [6] and vascular injury [7] whilst the role of the intracellular form (the only form expressed by ECs) is unclear.

We have previously reported that a genetic variant of IL-1ra is linked with reduced icIL-1ra1 production by EC [8] and a diminished proliferative capacity by EC [9]. Other studies have suggested a role for G1 cell cycle proteins in the IL-1 mediated proliferation of vascular smooth muscle cells [10] and the senescence of ECs [11]. Cyclin dependent kinase-2 (CDK2) activity is diminished in senescent HUVEC whilst bypassing senescence with telomerase restores CDK2 translation and activity [11].

In this study we report that a function of icIL-1ra1 in EC is to promote proliferation. Our data suggest that icIL-1ra1 mediates this effect in EC by IL-1R1 dependent binding, modulation of G_1 cell cycle proteins by increasing CDK2 activity and retinoblastoma protein phosphorylation. It is likely that expression of icIL-1ra could increase endothelial proliferation and thus, re-endothelialisation after vascular injury.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) and mouse lung endothelial cells (MLEC) were isolated and cultured using techniques based on previously described methods [12,13]. This study conforms to the principles outlined in the Declaration of Helsinki for the use of human tissue. Ethical approval for the isolation of cells from cords was given by the North Sheffield NHS Trust Local Research Ethics Committee 05/Q2308/17. IL-1ra null mice were a gift from Martin Nicklin (Sheffield) [14] and were cared for in accordance with the 1996 NIH Guide for Care and Use of Laboratory Animals. HUVEC were cultured in M199 media supplemented with 20% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L L-glutamine, 20 μg/mL endothelial cell growth factor (ECGF) and 95 μg/mL heparin. MLEC were cultured in DMEM supplemented with 20% FBS, penicillin, streptomycin, L-glutamine, non-essential amino acids, ECGF and heparin. EC between two and four passages were used for experiments and quiescence was achieved by serum deprivation for 24 h.

2.2. Adenoviral infection

HUVEC and MLEC were infected with adenoviral constructs containing an RSV promoter and encoding full-length human icIL-1ra1, sIL-1ra or GFP (Gene Transfer Vector Core, Iowa). Infection was at a multiplicity of infection (MOI) of 60 pfu/cell for 4 h in serum free media. Expression of IL-1ra was measured by Western blot (R&D Systems), and GFP was visualised by fluorescence microscopy or flow cytometry.

2.3. Proliferation and apoptosis assays

Proliferation and apoptosis of HUVEC or MLEC were measured by Ki67 immunoreactivity, ^{3}H thymidine incorporation, TUNEL staining and time lapse video microscopy as previously described [9,10,15]. Quiescent HUVEC or MLEC, which had been infected with the appropriate adenovirus construct, were stimulated with 1 ng/mL and 10 ng/mL

*Corresponding author. Fax: +44 114 2268898.
E-mail address: r.dewberry@sheffield.ac.uk (R.M. Dewberry).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.02.021

IL-1β (R&D Systems), 10 ng/mL and 100 ng/mL TNFα (Peprotech) or culture media alone for 48 h.

2.4. CDK2 assay and cell cycle protein detection

EC were synchronised to G_0 of the cell cycle by serum deprivation (24 h) and proliferation induced with M199 media (20% bovine serum) for 8 h. HUVEC were lysed with 50 mmol/L Hepes pH7.5, 250 mmol/L NaCl, 0.1% NP40, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 0.1 mmol/L sodium orthovanadate, 10 μg/mL aprotinin and 20 μmol/L leupeptin. Lysates were pre-cleared with protein-G sepharose beads and CDK2 immunoprecipitated (1 μg/mL anti-CDK2 antibody; Santa Cruz Biotechnologies). Beads were washed twice with lysis buffer and twice more with kinase buffer (50 mmol/L Tris–HCl (pH 7.5), 10 mmol/L $MgCl_2$, 1 mmol/L DTT). The beads were incubated with kinase buffer (supplemented with 0.5μmol/L ATP, 5 μg/mL histone-H1 and 3 μCi γ^{32}P ATP) at 30 °C for 30 mi. Phosphorylated histone-H1 was visualised by SDS–PAGE and exposure to X-OMAT X-ray film.

Cell cycle protein expression was detected by Western blot analysis using antibodies targeting CDK2 (Santa Cruz Biotechnologies), Cyclin E (Oncogene), $p21^{cip1/waf1}$ and Rb proteins (both BioSource). CDK2 activity and expression of cell cycle proteins were quantified by means of densitometry with Syngene software.

2.5. Quantification and statistics

Data were analysed using 2-tailed paired *t*-tests. A value of $P < 0.05$ were considered to be significant.

3. Results

3.1. ECs lacking IL-1ra have an enhanced rate of apoptosis

To support our earlier findings that HUVEC with a genetically determined programme to make low levels of icIL-1ra grow poorly in culture [9], we isolated MLEC from mice lacking all forms of IL-1ra and from wild-type littermate controls and analysed apoptosis under conditions of serum withdrawal using time lapse video microscopy (TLVM). There was an enhanced rate of apoptosis in MLEC lacking IL-1ra compared to wild-type MLEC over a 24 h period (Fig. 1A). Furthermore, MLEC isolated from IL-1RN$^{-/-}$ mice failed to thrive compared with controls (Fig. 1B and C) and were generally unable to survive beyond a second passage.

3.2. icIL-1ra1 suppresses the anti-proliferative effects of IL-1β in human EC and promotes cell growth

The ic form of IL-1ra is the only isoform produced by HUVEC [8,9] therefore, we examined, in detail, the role of icIL-1ra1 upon human EC proliferation by using adenoviral constructs containing icIL-1ra1 or GFP. Over-expression of icIL-1ra1 by EC was detected at 2, 4 and 8 days post-infection with 60 pfu/cell Ad5RSVicIL-1ra (Fig. 2A) with a transfection efficiency of >90% determined by the fraction of cells expressing GFP at 4 days post-infection with 60 pfu/cell Ad5RSVGFP (Fig. 2B).

In subsequent experiments at 4 days post-infection with 60 pfu/cell Ad5RSVicIL-1ra1 or Ad5RSVeGFP, EC were quiesced for 24 h in media containing 2% bovine serum and stimulated with 1 ng/mL or 10 ng/mL IL-1β for 48 h. EC proliferation (determined by Ki67 expression) decreased in a dose-dependent manner with the Ad5RSVeGFP control, this response was inhibited by over-expression of icIL-1ra1 (Fig. 3A).

To determine if this was specifically an IL-1 dependent mechanism, the anti-proliferative effect of TNFα upon EC proliferation was also measured (Fig. 3B). Over-expression of icIL-1ra1 had no significant effect upon the proliferation of TNFα stimulated cells.

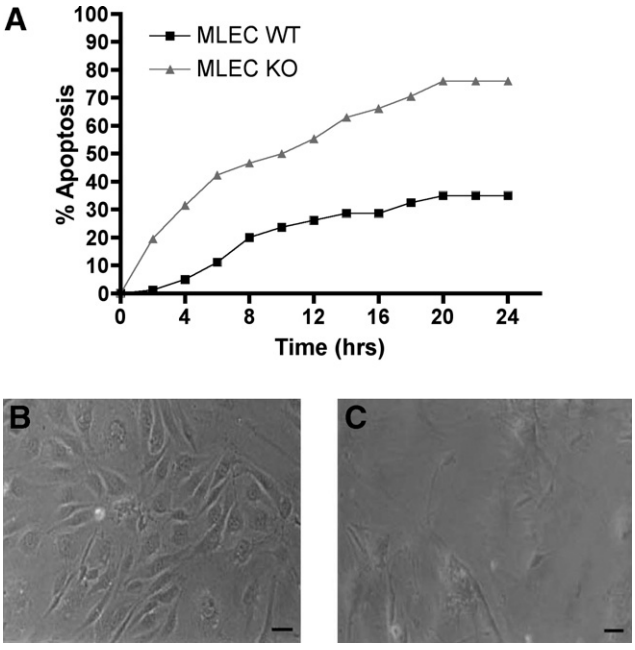

Fig. 1. Apoptosis in murine lung endothelial cells (MLEC) from IL-1RN −/−mice and wild-type littermate controls. (A) Representative cumulative apoptotic index over 24 h for MLEC from IL-1RN−/− (KO) and wild-type animals. (B and C) Phase contrast micrographs of MLEC from wild-type and IL-1RN−/− mice respectively at first passage. Note the sparsity and senescent appearance of cells in (C) originally seeded at the same density as cells in (B). Scale bar = 5 μm.

We have previously shown that icIL-1ra is released from endothelial cells [16,17]. To establish if modulation of cell cycle proteins by EC icIL-1ra is dependent upon binding to the cell-surface type 1 IL-1 receptor (IL-1R1), we measured ^{3}H thymidine incorporation in IL-1R1 deficient and wild-type MLEC following infection with 60 pfu/cell Ad5RSVicIL-1ra1 or Ad5RSVeGFP. Proliferation was significantly enhanced in wildtype MLEC that expressed icIL-1ra1 compared to the GFP controls ($P = 0.0215$). There was no change in the proliferation of IL-1R1 null MLEC under similar conditions (Fig. 3C).

3.3. icIL-1ra1 induces CDK2 activity and retinoblastoma phosphorylation in ECs

Regulation and expression and activity of G_1 and S phase cell cycle proteins by over-expression of icIL-1ra was studied by measuring histone-H1 phosphorylation. EC were shown to be synchronised at G_0 following serum deprivation and then at G_1/S phase following addition of EC culture media for 8 h by measuring DNA content using propidium iodide and flow cytometry (data not shown). EC expressing icIL-1ra1 showed greater histone-H1 phosphorylation compared with cells expressing GFP and uninfected controls (Fig. 4A). Analysis of cell cycle proteins by Western blot showed no significant changes of cyclin E, CDK2 and $p21^{cip1/waf1}$ levels with icIL-1ra1 expression as quantified by densitometry (Fig. 4B).

To determine if enhanced CDK2 activity had a role in downstream cell cycle signalling we measured retinoblastoma

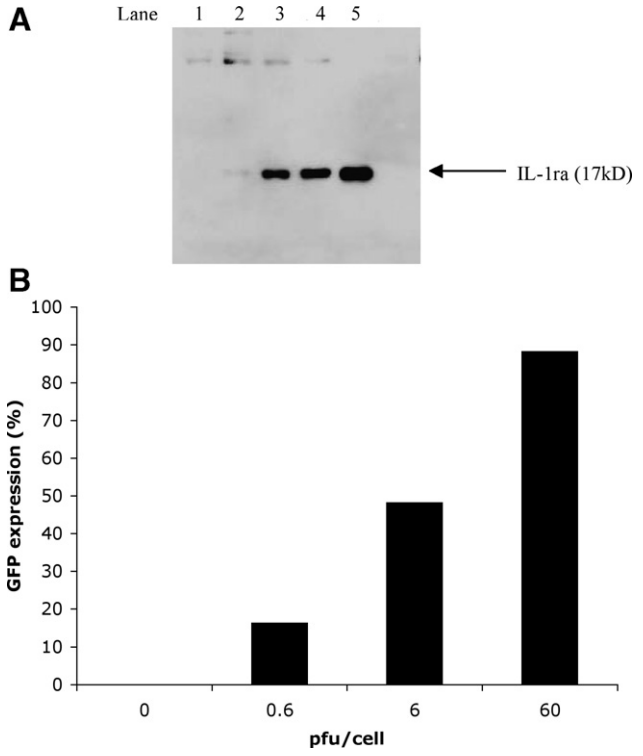

Fig. 2. Time-course and dose response of EC following infection with Ad5RSVicIL-1ra1 or Ad5RSVGFP. (A) Time-course of icIL-1ra protein expression by Western blot analysis 2, 4 and 8 days post-infection (lanes 2–4). Wells were loaded for equal protein. Lanes 1 and 5 are unstimulated EC and hrIL-1ra, respectively. (B) Expression of GFP protein by EC following infection with Ad5RSVGFP at 0, 0.6, 6 and 60 pfu/cell. The percentage of GFP expressing cells were measured by flow cytometry analysis from a total of 10000 events. A representative experiment is shown.

protein phosphorylation using an anti-Rb antibody that recognises phosphorylated and non-phosphorylated forms. Hyper-phosphorylation of Rb mediates the G_1 to S phase transition of the cell cycle by release of the transcription factor E2F. As expected, given the increase in CDK2 activity (Fig. 4A), we observed predominantly hyper-phosphorylated Rb in EC over-expressing icIL-1ra1 and sIL-1ra. This is in comparison to uninfected and GFP controls in which both phosphorylated and non-phosphorylated forms of Rb were present (Fig. 4).

4. Discussion

The data presented here indicate a potential mechanism for icIL-1ra involvement in EC proliferation. We deduce that one likely mechanism of action for icIL-1ra1 may be via its release from EC and subsequent binding to cell-surface IL-1R1.

The ability of icIL-1ra1 to overcome a decrease in proliferation in EC induced by IL-1 is consistent with recent data in IL-1ra deficient mouse fibroblasts [18] and in MLEC (shown here) where IL-1ra null cells proliferate less well and undergo apoptosis at an increased rate compared to those studied at an identical timepoint from wild-type littermates. An increase in p38 and $p16^{INK4a}$ activation has also been shown [18] suggesting that traversing the cell cycle is not possible in IL-1ra null fibroblasts. Conversely, over-expression of icIL-1ra in human intestinal epithelial cells and keratinocytes inhibited p38 activation [19]. In keratinocytes, this effect was mediated by icIL-1ra1 interacting with the COP9 signalosome (CSN3) [20]. The

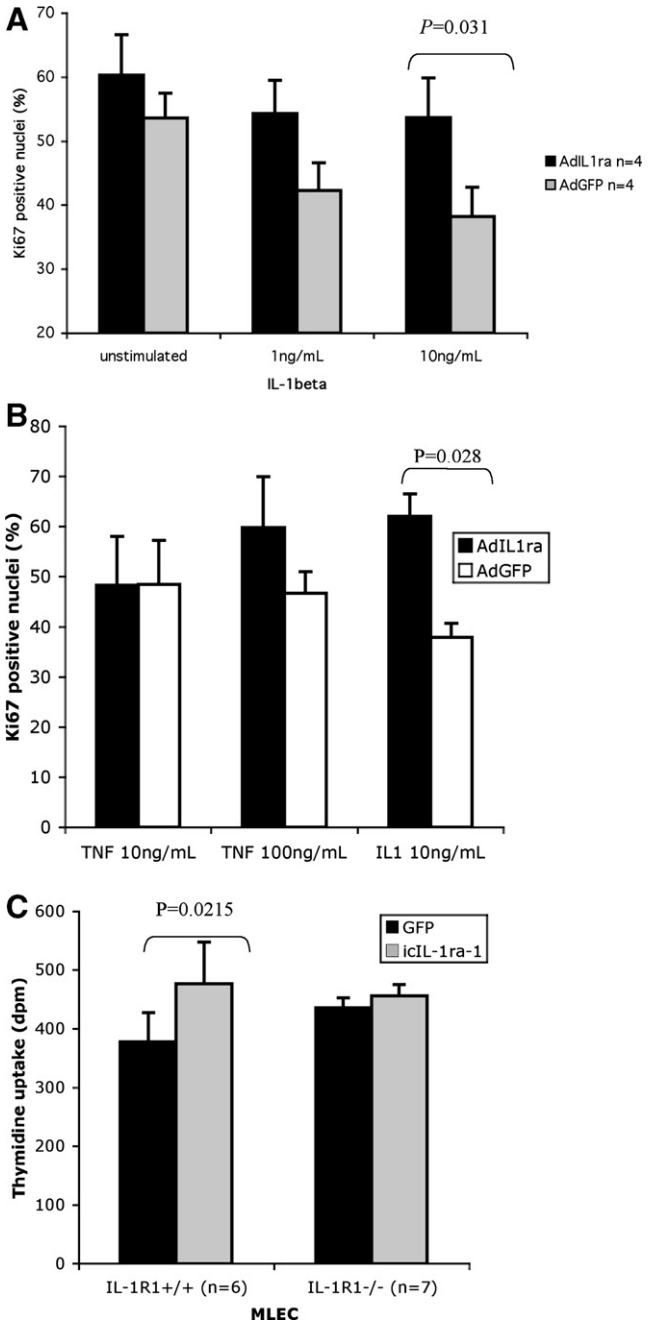

Fig. 3. icIL-1ra1 abrogates the anti-proliferative effect of IL-1β upon EC. (A) Quiescent EC stimulated for 48 h with IL-1β (1 ng/mL or 10 ng/mL) 4 days post-infection with Ad5RSVicIL-1ra-1 or Ad5RS-VeGFP ($n = 4$). Proliferation was measured as the percentage of Ki67 positive nuclei (means ± S.E.M.). Statistical analysis was a paired t-test ($P = 0.031$). (B) HUVEC expressing icIL-1ra or GFP and stimulated with 10 ng/mL or 100 ng/mL TNFα, proliferation was measured by Ki67 detection and expressed as an index of unstimulated EC ($n = 3$). Stimulation with 10 ng/mL IL-1β was used as a control. (C) IL-1R1 deficient MLEC expressing icIL-1ra1 or GFP (60 pfu/cell) and proliferation measured by ^{3}H thymidine incorporation. Data are the means of triplicate measurements and statistical analysis was a paired t-test.

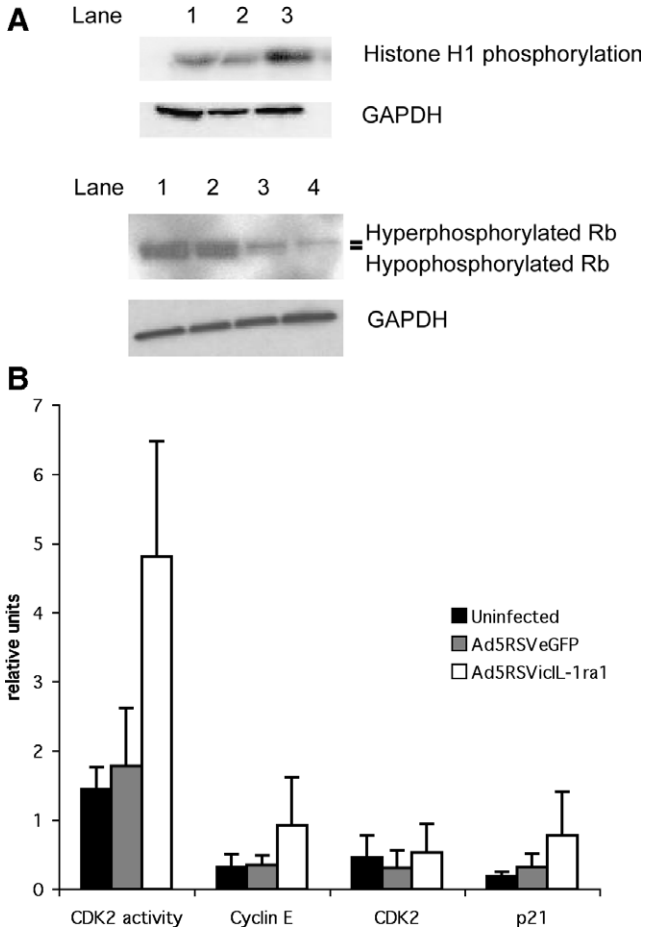

Fig. 4. icIL-1ra1 enhances CDK2 activity by EC. (A) EC expressing GFP or icIL-1ra1 (60 pfu/cell) and synchronised to G_0 were induced to proliferate with M199 growth media for 8 h. CDK2 activity was measured by histone-H1 phosphorylation (lanes 1–3 are control, GFP and icIL-1ra1 transfected cells, respectively). Rb phosphorylation was measured using a phospho-specific antibody; the upper and lower bands are hyperphosphorylated and hypophosphorylated Rb respectively (lanes 1–4 are control, GFP, icIL-1ra1 and sIL-1ra). Equal loading is indicated by GAPDH expression. (B) Quantification of Histone H1 Cyclin E, CDK2, p21 and GAPDH band intensities were measured by densitometric analysis. Data (means ± S.E.M.) are relative units compared with GAPDH expression ($n = 2$).

consequences upon cell functions such as proliferation or senescence were not studied further by these authors.

In contrast to the aforementioned studies [18,19], our work is the first to link the function of icIL-1ra1 to events beyond cytoplasmic protein kinase activation, to control of the cell cycle in human EC, and, hence, plausibly to the pathogenesis of cardiovascular disease. These EC data contrast with the role of IL-1 in VSMCs, where IL-1 enhances platelet-derived growth factor-BB (PDGF-BB) cell proliferation, an effect mediated by increased CDK2 activity [10]. Administration of IL-1ra to these cells inhibits VSMC proliferation whilst IL-1ra in combination with PDGF-BB enhances proliferation [10].

The data presented here indicate a potential mechanism for icIL-1ra involvement in EC proliferation by promoting cell cycle transition from G_1 to S phase. It is likely that this is mediated in several ways. First, icIL-1ra1 may be released from EC to act upon the external IL-1 receptor [16]. Second, icIL-1ra1 may act via an undefined mechanism inside the cell to increase transcription of cell cycle proteins such as cyclin D in much the same way that has been proposed for IL-1α [21]. Third, icIL-1ra1 could interact with signalling platforms such as CSN3 regulating multiple kinase pathways and cytokine-regulated cell outputs [20].

Given the data presented here, that icIL-1ra1 does not modulate the proliferation of IL-1R1 null EC, we suggest that at least in this experimental setting, icIL-1ra1 modulates proliferation by being released from EC and interacting with the cell-surface IL-1R1 receptor. We have previously shown the importance of IL-1R1 in the biological actions of icIL-1ra by its ability to inhibit inflammatory signalling independently of the known IL-1 intracellular signalling pathway [22]. In addition, these data support the finding that, in a mouse model of dermal inflammation, icIL-1ra1 mediated effects were dependent upon IL-1R1 [23]. However, this does not necessarily exclude the involvement of other intracellular mechanisms that may also be operating at lower levels or in response to different micro-environmental stimuli and/or in other cell types.

In summary, the data presented here indicate that icIL-1ra1 increases EC proliferation by promoting CDK2 activity and that this is dependent upon binding to the IL-1R1. We show also that augmentation of icIL-1ra1 protein levels can control EC growth via cell cycle dependent mechanisms. This is likely to be a useful and important mechanism of action for IL-1ra which could be of potential therapeutic value contributing to EC protection, re-growth and thus healing in vessels after vascular injury.

Acknowledgements: The authors thank Amgen for IL-1ra and Dr. Martin Nicklin, University of Sheffield for IL-1RN mice and littermate controls.

References

[1] Ross, R. (1999) Atherosclerosis is an inflammatory disease. Am. Heart J. 138 (5 Pt 2), S419–S420.
[2] Garfinkel, S. et al. (1994) Post-transcriptional regulation of interleukin 1 alpha in various strains of young and senescent human umbilical vein endothelial cells. Proc. Natl. Acad. Sci. USA 91 (4), 1559–1563.
[3] Maier, J.A. et al. (1990) Extension of the life-span of human endothelial cells by an interleukin-1 alpha antisense oligomer. Science 249 (4976), 1570–1574.
[4] Sawada, H., Kan, M. and McKeehan, W.L. (1990) Opposite effects of monokines (interleukin-1 and tumor necrosis factor) on proliferation and heparin-binding (fibroblast) growth factor binding to human aortic endothelial and smooth muscle cells. In Vitro Cell Dev. Biol. 26 (2), 213–216.
[5] Muzio, M. et al. (1999) Characterization of type II intracellular IL-1 receptor antagonist (IL-1ra3): a depot IL-1ra. Eur. J. Immunol. 29 (3), 781–788.
[6] Elhage, R. et al. (1998) Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. Circulation 97 (3), 242–244.
[7] Chamberlain, J. et al. (2006) Interleukin-1beta and signaling of interleukin-1 in vascular wall and circulating cells modulates the extent of neointima formation in mice. Am. J. Pathol. 168 (4), 1396–1403.
[8] Dewberry, R. et al. (2000) Interleukin-1 receptor antagonist expression in human endothelial cells and atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 20 (11), 2394–2400.
[9] Dewberry, R.M., Crossman, D.C. and Francis, S.E. (2003) Interleukin-1 receptor antagonist (IL-1RN) genotype modulates the replicative capacity of human endothelial cells. Circ. Res. 92 (12), 1285–1287.

[10] Nathe, T.J. et al. (2002) Interleukin-1beta inhibits expression of p21(WAF1/CIP1) and p27(KIP1) and enhances proliferation in response to platelet-derived growth factor-BB in smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 22 (8), 1293–1298.
[11] Freedman, D.A. and Folkman, J. (2005) CDK2 translational down-regulation during endothelial senescence. Exp. Cell Res. 307 (1), 118–130.
[12] Jaffe, E.A. et al. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52 (11), 2745–2756.
[13] Dong, Q.G. et al. (1997) A general strategy for isolation of endothelial cells from murine tissues. Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. Arterioscler. Thromb. Vasc. Biol. 17 (8), 1599–1604.
[14] Nicklin, M.J. et al. (2000) Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. J. Exp. Med. 191 (2), 303–312.
[15] King, A.R. et al. (2003) A role for caspase-1 in serum withdrawal-induced apoptosis of endothelial cells. Lab. Invest. 83 (10), 1497–1508.
[16] Wilson, H.L. et al. (2004) Secretion of intracellular IL-1 receptor antagonist (type 1) is dependent on P2X7 receptor activation. J. Immunol. 173 (2), 1202–1208.
[17] Wilson, H.L. et al. (2007) P2X receptor characterization and IL-1/IL-1Ra release from human endothelial cells. Br. J. Pharmacol.
[18] Uekawa, N. et al. (2004) Involvement of IL-1 family proteins in p38 linked cellular senescence of mouse embryonic fibroblasts. FEBS Lett. 575 (1–3), 30–34.
[19] Garat, C. and Arend, W.P. (2003) Intracellular IL-1Ra type 1 inhibits IL-1-induced IL-6 and IL-8 production in Caco-2 intestinal epithelial cells through inhibition of p38 mitogen-activated protein kinase and NF-kappaB pathways. Cytokine 23 (1–2), 31–40.
[20] Banda, N.K. et al. (2005) Intracellular IL-1 receptor antagonist type 1 inhibits IL-1-induced cytokine production in keratinocytes through binding to the third component of the COP9 signalsome. J. Immunol. 174, 3608–3616.
[21] Werman, A. et al. (2004) The precursor form of IL-1alpha is an intracrine pro-inflammatory activator of transcription. Proc. Natl. Acad. Sci. USA 101 (8), 2434–2439.
[22] Evans, I.D.S., Francis, S.E., Crossman, D.C. and Wilson, H.L. (2006) Action of icIL-1ra is independent of IL-1 intracellular signalling pathway. Cytokine 33 (5), 274–280.
[23] Palmer, G. et al. (2007) Type I IL-1 receptor mediates IL-1 and intracellular IL-1 receptor antagonist effects in skin inflammation. J. Invest. Dermatol. 127 (8), 1938–1946.