Atherosclerosis 236 (2014) 108–115

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Angiotensin receptor 1 blockade reduces secretion of inflammation associated cytokines from cultured human carotid atheroma and vascular cells in association with reduced extracellular signal regulated kinase expression and activation

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ARTICLE INFO

Article history:
Received 5 April 2014
Received in revised form
21 May 2014
Accepted 11 June 2014
Available online 28 June 2014

Keywords:
Anti-Inflammatory
Angiotensin II
Angiotensin receptor blocker
Extracellular signal regulated kinase
Atheroma
Cytokine

ABSTRACT

Background: A number of studies have suggested that angiotensin II (AII) receptor type 1 (ATR1) blocking drugs (ARBs) have anti-inflammatory effects however the mechanisms responsible are poorly investigated.
Objective: To determine the role of extracellular signal regulated kinase (ERK)1/2 in ARB induced anti-inflammatory effects within human carotid atherosclerosis.
Methods: Atheroma samples obtained from patients undergoing carotid endarterectomy were cultured with and without ATR1 (irbesartan), ERK1/2 (PD98059), AII ([Sar^1, Ile^8]-AII) and angiotensin converting enzyme (ACE)2 (DX600) blockade. The *in vitro* effects of ATR1 and ERK1/2 blockade and exogenous AII on serum stimulated healthy, primary vascular cells were also investigated. Outcome was assessed by measuring cytokine, (interleukin (IL)-6, IL-8, C–C motif chemokine (CCL)2, C-X-C motif chemokine (CXCL)5, osteoprotegerin (OPG), osteopontin (OPN), CXCL16), concentrations in supernatants and phosphorylated ERK1/2 in the tissue lysates using ELISA. ERK1/2 expression in the tissue was assessed using Western blotting.
Results: Irbesartan reduced concentrations of IL-6, IL-8, CCL2, CXCL5, OPG, OPN and CXCL16 in both atheroma and primary vascular cell culture supernatants. The reduction in cytokine levels in the atheroma supernatant was correlated to a reduction in ERK1/2 expression in the tissue. Inhibition of ERK1/2 downregulated IL-6, IL-8 and CXCL5 in both atheroma and cell culture supernatants. AII and ACE2 blockade had no impact on cytokine or active ERK1/2 levels in the atheroma culture.
Conclusion: Our findings suggest that ATR1 blockade downregulates atheroma tissue ERK1/2 expression leading to a reduction in cytokine production and that a non-AII agonist ATR1 signalling response may induce expression of these inflammation associated cytokines in the atheroma.

1. Introduction

The renin-angiotensin signalling pathway plays a key role in regulating the cardiovascular system. It is widely accepted that angiotensin II (AII) signalling through angiotensin receptor (ATR)1 (Fig. 1) promotes cardiovascular events. Treatment with ATR1 blocking drugs (ARBs) has been associated with a reduction in cardiovascular events, such as stroke, in a number of clinical trials [1–3]. These studies suggest ARBs have an impact beyond their anti-hypertensive actions [1,3] and can directly reduce inflammation associated with cardiovascular disease (CVD) [4–5] with reductions in levels of a range of inflammatory cytokines in patients' receiving ARB therapy [6–15]. AII/ATR1 signalling also leads to the activation of several downstream kinase pathways, such as

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http://dx.doi.org/10.1016/j.atherosclerosis.2014.06.011
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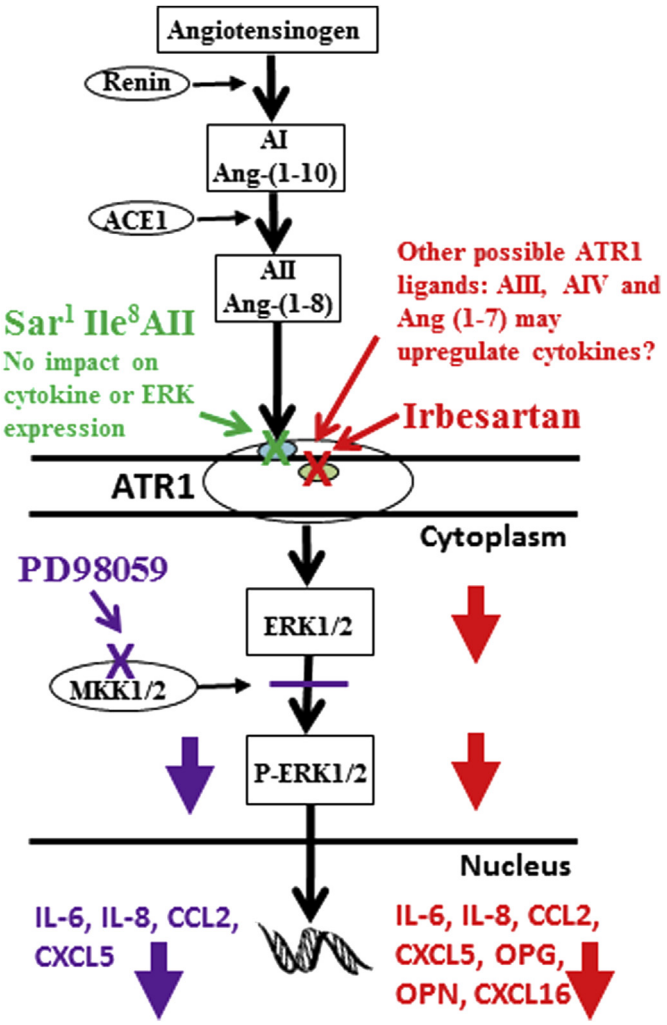

Fig. 1. The angiotensin II/angiotensin type 1 receptor signalling pathway. Presented is a simplified pathway by which ATR1 activation potentially leads to inflammatory cytokine production. ATR1 has two different, distinct binding sites, an extracellular pocket for the angiotensin peptides (blue) and a separate membrane binding site (green) for the non-peptide diphenylimidazole antagonists, such as irbesartan (red cross). AII binding was blocked by the specific peptide antagonist, [Sar[1], Ile[8]]-AII (green cross), which binds directly to the peptide binding pocket (blue). Activation of ERK1/2 was blocked by PD98059, which binds to MKK1/2, inactivating the enzymes capacity to phosphorylate ERK1/2 (purple cross). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extracellular signal regulated kinases (ERK)1/2 [16], which are also associated with inflammatory cytokine upregulation [17–18].

Examining the cellular signalling responses that lead to the anti-inflammatory effects of ATR1 blockade in patients is not straight forward. One approach is the use of tissue explant culture [19–22]. This culture system has been demonstrated to simulate the *in vivo* AII response to ARB [6,23–24] with an increase in AII levels in the atheroma explant culture supernatant with ATR1 blockade [19].

In this study we aimed to investigate the impact of ATR1 and ERK1/2 blockade on inflammatory cytokine production *in vitro* using two different culture systems. The first system utilised cultured, diseased, human carotid atheroma tissue [19–22]. The second system used healthy, primary vascular cells stimulated by serum [22]. Previous studies have demonstrated that exposure of smooth muscle cells (SMC) or fibroblasts to blood initiates cell signalling responses, such as inflammatory cytokine release, within the vasculature [25].

There are a number of studies reporting that the ATR1 can be stimulated by non-AII agonists, which are also inhibited by ARBs [26–29]. A secondary element of this study was to investigate the role of AII in promoting inflammatory cytokine secretion from atheroma. The effect of an AII peptide antagonist [30–35] and an angiotensin converting enzyme (ACE)2 inhibitor on inflammatory cytokine secretion were also investigated. ACE2 is the carboxypeptidase which converts AII to Ang-(1–7). The authors demonstrated previously that blockade of ACE2 activity in atheroma increased AII levels ~10-fold *in vitro* [19].

The aims of this study were threefold: Firstly, to clarify the role of ERK1/2 in the ability of ATR1 blockade to limit inflammation in human carotid atheroma, secondly to investigate the role of AII in stimulating inflammation in human carotid atheroma and thirdly, to determine if ATR1 and ERK1/2 blockade had anti-inflammatory effects in human, primary vascular cells stimulated by serum.

2. Methods

2.1. Patients

Patients were recruited from those selected to undergo primary carotid endarterectomy at The Townsville and Mater Hospitals, Queensland, Australia between Jul 2011 and Feb 2013. Inclusion criteria included: 1) verbal and written informed consent; 2) carotid stenosis of ≥70% identified on duplex imaging using criteria previously described [20]; and 3) no previous carotid artery intervention. Ethics approval was provided by the appropriate committees (Townsville Health Service District, 61/05). Patients with a history of transient ischaemic attack (TIA), stroke with good recovery and those with an asymptomatic stenosis were included. Please refer to Appendix A online for the patient characteristics (http://atherosclerosis-journal.com).

2.2. Explant culture

Explant culture was carried out as previously described [19–22]. Two macroscopically similar, disease matched, atheroma biopsy pairs were obtained from each patient. The interventional agent concentrations were based on circulating concentrations measured in patients receiving therapeutic levels or published effective *in vitro* concentrations. Therapeutic circulating concentrations of irbesartan are reported to be between ~1 and 10 mmol/L [36–37]. The MKK1/2 inhibitor, PD98059, binds directly to MKK1/2 blocking its capacity to phosphorylate ERK1/2, consequently blocking ERK1/2 activation [38]. When used at a concentration of 20 μmol/L, PD98059, abolished AII induced upregulation of C-reactive protein from vascular SMCs [39]. The AII peptide antagonist, [Sar[1], Ile[8]]-AII, has been used in culture at a variety of concentrations ranging from 0.1 μmol/L [30–31], 1 μmol/L [32–34] to 10 μmol/L [35].

The following interventions were assessed: a) ATR1 (Irbesartan; 2 mmol/L; Sigma #I2286); b) ERK1/2 (PD98059; 20 μmol/L; Sigma #P215); c) AII ([Sar[1], Ile[8]]-AII; 10 μmol/L; Auspep #2153); d) ACE2 (DX600; 1 μmol/L; Anaspec #62337) [19].

2.3. Primary vascular cell culture

Due to the nature of the carotid endarterectomy procedure the types of vascular cells present in the explant tissue include: endothelial cells (ECs), SMCs, inflammatory cells which have infiltrated the atheroma from the blood and red blood cells (RBCs) on the tissue surface. A previously developed mixed, healthy, primary vascular cell technique [22] was used to assess the impact of ATR1 and ERK1/2 activity blockade, and increasing levels of AII on inflammatory cytokine production from vascular cells stimulated by