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Co-localization of angiotensin-converting enzyme 2-, octomer-4- and CD34-positive cells in rabbit atherosclerotic plaques

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Angiotensin-converting enzyme 2 (ACE2) is a novel enzyme with possible implications in the treatment of blood pressure disorders. Recent evidence suggests that an upregulation of ACE2 can be stimulated by all-*trans* retinoic acid (at-RA); however, at-RA also affects regulation of the stem-cell marker octomer-4 (Oct-4) and thus cellular differentiation. We have previously shown that smooth muscle cells and macrophages present within rabbit atherosclerotic plaques are positive for ACE2, Oct-4 and the haematopoietic stem-cell marker CD34. Thus, to provide evidence that possible at-RA treatment could affect both plaque cellular biology (via effects on cellular differentiation) and blood pressure (via ACE2), it is vital to show that cells with atherosclerotic plaques co-express all three markers. Thus, we sought to provide evidence that a subset of cells within atherosclerotic plaques is positive for ACE2, Oct-4 and CD34. We used New Zealand White rabbits that were fed a control diet supplemented with 0.5% cholesterol plus 1% methionine for 4 weeks and then allowed to consume a normal diet for 10 weeks. Immunohistochemistry was performed by standard techniques. We report that ACE2, Oct-4 and CD34 were all present within atherosclerotic plaques. Although macrophages were positive for all three markers, spindle-shaped cells in the media did not show all three markers. The endothelium overlying normal arterial wall showed positive Oct-4 and ACE2 immunoreactivity, but CD34 immunoreactivity was patchy, indicating that such cells might not have fully differentiated. It is concluded that cells in atherosclerotic plaques express co-express ACE2, Oct-4 and CD34. Further studies aimed at establishing the effects of all-*trans* retinoic acid on blood pressure and atherosclerotic cell differentiation are warranted.

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Angiotensin-converting enzyme inhibitors (ACEi) and antagonists of angiotensin II receptors are successful medications for the treatment of cardiovascular diseases (CVD). This has now established an overactive renin–angiotensin system (RAS) as a major culprit of CVD and hypertension (Paul *et al.* 2006). Current research suggests that the heptapeptide, Ang(1–7), has a beneficial role in the prevention of CVD, and this has been further substantiated by the recent discovery of angiotensin-converting enzyme 2 (ACE2), the major enzyme involved in the formation of Ang(1–7). It is well documented that the actions of the ACE2–Ang(1–7) axis counterregulate the ACE–angiotensin II axis (Santos *et al.* 2005; Ferrario, 2006; Reudelhuber, 2006), and some studies have suggested that the activation of the ACE2–Ang(1–7) axis can prevent or reduce the damage observed in CVD (Loot *et al.* 2002; Diez-Freire *et al.* 2006; Ferreira *et al.* 2007). Indeed, an increase in plasma Ang(1–7) is observed after chronic treatment with ACEi and/or angiotensin II receptor antagonists, suggesting that it could be possible that the beneficial effects of these RAS blockers result, in part, from Ang(1–7)-mediated effects (Chappell *et al.* 1998; Iyer *et al.* 1998). Therefore, the emerging concept is that an imbalance in Ang(1–7)–angiotensin II levels is critical in the development of CVD.

Angiotensin-converting enzyme 2 was discovered by two independent research groups in 2000 (Donoghue *et al.* 2000; Tipnis *et al.* 2000). It is an 805-amino-acid

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ACE homologue and has 42% identity with ACE in the catalytic domain (Donoghue *et al.* 2000; Tipnis *et al.* 2000); however, ACE2 is resistant to ACE inhibitors such as lisinopril, captopril and enalaprilat (Guy *et al.* 2003). The organ distribution of ACE2 includes the endothelium of the heart, kidney and testis (Donoghue *et al.* 2000), myocytes, lung epithelial cells, vascular smooth muscle cells, ileum, duodenum, jejunum, caecum, colon (Harmer *et al.* 2002) diseased human arteries (Zulli *et al.* 2007*a*) and rabbit atherosclerotic arteries (Zulli *et al.* 2006).

As an adjunct treatment for high blood pressure, all-*trans* retinoic acid (at-RA) has recently been used to lower blood pressure in hypertensive animals via the stimulation of ACE2. All-*trans* retinoic acid is known to influence gene expression and protein production in many ways. A single, classical pathway for the way in which at-RA works has emerged, consisting of all-*trans* retinoic acid, plus a dimer of retinoic acid receptor (RAR) and retinoid X receptor (RXR; Zhong *et al.* 2004). Recent studies have shown that at-RA can influence gene expression within the RAS, which plays a role in the pathogenesis of hypertension (Zhong *et al.* 2004). The beneficial effects of at-RA are generally exerted via interference with the action of the RAS (Dechow *et al.* 2001). For example, Zhong *et al.* (2004) showed that spontaneously hypertensive rats (SHR) treated with at-RA significantly increased the expression of ACE2 mRNA and protein, which was also accompanied by a reduction in blood pressure. However, chronic treatment with at-RA on non-hypertensive rats did not have any effect on the expression level of ACE2 (Hamming *et al.* 2007).

Therefore at-RA could potentially be used to treat hypertensive patients, but at-RA can also affect cellular differentiation by downregulating the transcription factor octomer-4 (Oct-4), which is also known as Oct3/NF-A3/POU5F1 (Scholer *et al.* 1989; Ovitt & Scholer, 1998). Octomer-4 a member of the POU (Pituitary-specific Pit-1, Octamer transcription factor sequence - ATGCAAAT and the neural Unc-86 transcription factor) domain family of octamer-binding proteins (Ovitt & Scholer, 1998). Scholer *et al.* (1989) first identified Oct-4 in embryonic stem cells and embryonal carcinoma cells. Octomer-4 has only been found in mammals. The human Oct-4 amino-acid sequence is 87% identical to mouse Oct-4. Octomer-4 is a maternally expressed octamer-binding protein, which is expressed specifically in the female germline at later stages of development (Scholer *et al.* 1989). Although the presence of maternally expressed Oct-4 is seen primarily in undifferentiated embryonic stem cells, unfertilized oocytes and primordial germ cells (Sylvester & Scholer, 1994), we have recently shown that cells expressing Oct-4 are also present in diseased human arteries (Zulli *et al.* 2007*c*) and rabbit atherosclerotic plaques (Zulli *et al.* 2007*b*), indicating that primitive cells expressing Oct-4 persist into adulthood and disease.

Thus, treatments to increase the expression levels of ACE2 may seem to be beneficial; however, the effects of at-RA on Oct-4 could also stimulate cellular differentiation. We therefore sought to provide evidence that cells within atherosclerotic plaques express both ACE2 and Oct-4 (as well as CD34) and thus provide the foundation for further studies into the effects of at-RA on both hypertension and atherosclerosis.

Methods

Male New Zealand White rabbits at 3 months of age ($n = 4$) were fed an atherogenic diet of normal rabbit chow diet supplemented with 0.5% cholesterol plus 1% methionine plus 5% peanut oil (Zulli *et al.* 2003, 2004) for 4 weeks followed by a normal diet for 10 weeks to induce fibrous cap formation. The atherogenic feeding time was shortened to 4 weeks in this study because 12 week atherogenic feeding times lead to liver dysfunction. At this time point (4 weeks), the abdominal aorta has abundant macrophage/foam cell deposition over the endothelium. The normal diet regimen is introduced to allow the further differentiation of cells. The animals were housed in individual cages and maintained at a constant temperature of approximately 21°C. Food and water were supplied *ad libitum*. The experiments were carried out according to the National Health and Medical Research Council *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (6th edn, 1997). The animals were killed with a bolus dose of xylazine (6 mg kg^{-1}) and ketamine (20 mg kg^{-1}) as previously described in our laboratory (Zulli *et al.* 2005, 2006). A 40 mm segment of abdominal aorta near the bifurcation was excised, cleaned of connective tissue and fat, cut into 5 mm rings, and fixed in freshly prepared 4% paraformaldehyde solution in PBS overnight. Sections were processed for paraffin and mounted on a single paraffin block to keep uniform cutting thickness and immunohistochemistry procedures constant.

Immunohistochemistry

Sections were randomly selected, dewaxed, rehydrated and placed in 10 mM TrisCl (pH 7.4). Sections were pre-incubated with 1% goat serum in 10 mM TrisCl (pH 7.4) for 20 min before incubating with the primary antibody diluted in 1% goat serum in 10 mM TrisCl (pH 7.4). Mouse monoclonal immunoglobulin G against ACE2 (catalogue no. ALX 804-715, diluted 1:150, Alexis Biochemicals, San Diego, CA, USA), mouse monoclonal immunoglobulin G against Oct-4 (ESC Kit no. SCR002, diluted 1:100, Chemicon International, Millipore Corporation, North Ryde, Australia) and mouse monoclonal immunoglobulin G against CD34 (CD34 Class III no. CBL 555, diluted 1:100, Chemicon

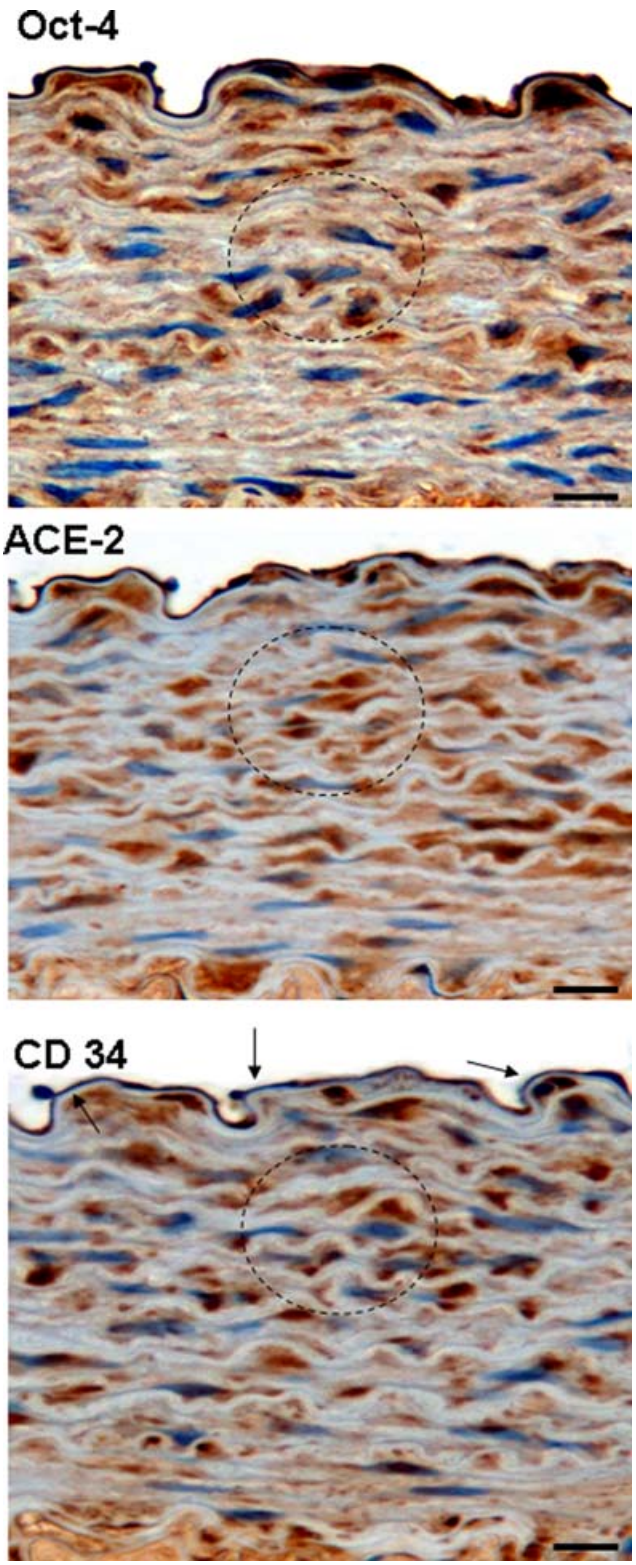

Figure 1. Photomicrographs of serial adjacent sections of rabbit abdominal aorta demonstrating Oct-4, ACE2 and CD34 immunoreactivity
Octomer-4, ACE2 and CD34 immunoreactivity was clearly evident in endothelium overlying normal arterial wall. As shown by arrows, CD34 immunoreactivity was not uniform, since blue (Haematoxylin) staining is prominent rather than the brown (diaminobenzidine). Cells in the media are positive for all three factors, although it was difficult to observe cells that co-localized all three proteins (dashed circles). Scale bars represent 25 μm.

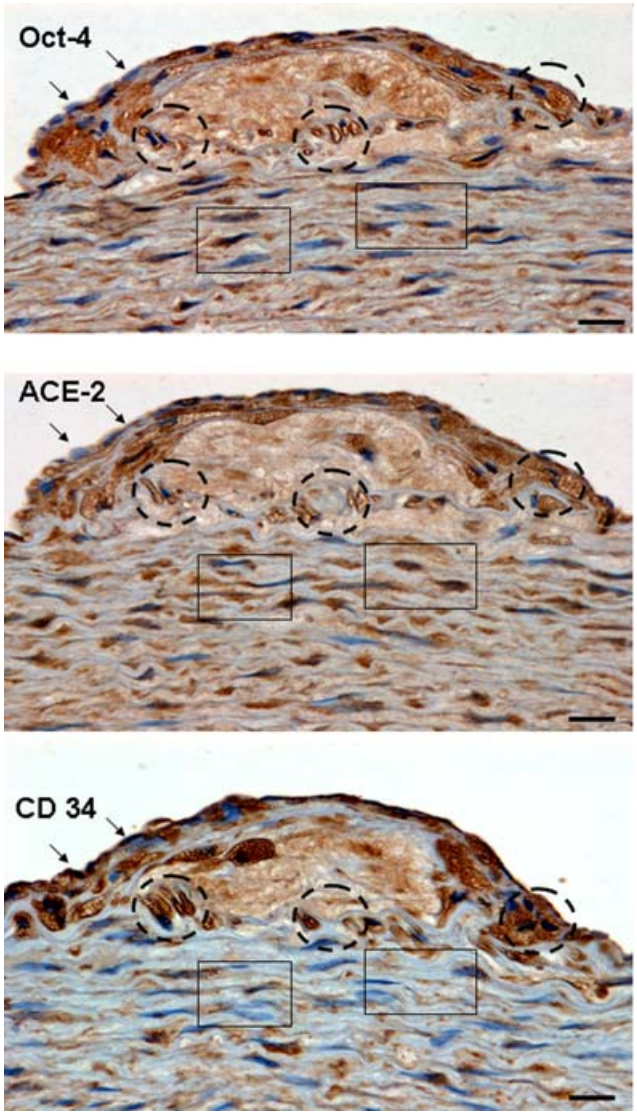

Figure 2. Photomicrographs of serial adjacent sections of rabbit abdominal aorta demonstrating Oct-4, ACE2 and CD34 immunoreactivity in small atherosclerotic plaques
Octomer-4, ACE2 and CD34 immunoreactivity was clearly evident in endothelium overlying plaques; however, some areas were negative for ACE2 and Oct-4 but positive for CD34 (arrows). In addition, macrophages and spindle-shaped cells (dashed circles) show co-expression of factors. In the media, specific spindle-shaped cells were visible for Oct-4, ACE2 or CD34 (boxes); however, it was difficult to determine co-localization. Scale bars represent 25 μm.

International) were incubated overnight. As a negative control, a monoclonal antibody to *Aspergillus niger* glucose oxidase (Dako) was diluted 1:20 and also incubated overnight. Immunohistochemistry was performed using

the 'Envision' commercially available kit, following the manufacturer's directions (catalogue no. K4001, DAKO, Carpentaria, CA, USA; Zulli *et al.* 2005, 2006, 2007*b*). Antigenic sites were developed with diaminobenzidine, counterstained with Haematoxylin, dehydrated and mounted with DPX mounting media (BDH, Poole, UK).

Results

Octomer-4, ACE2 and CD34 were clearly visible on the endothelial layer overlying a section of normal vessel wall in this atherosclerosis model (Fig. 1). However, in some areas, endothelial CD34 immunoreactivity was not expressed in the same locations as ACE2 and Oct-4 in serially adjacent sections (as indicated by arrows in Fig. 1). In addition, in the media, there appeared to be a lack of uniformity with staining of all three factors (dashed circles). To determine whether there were differences in cellular expression of all three factors between small and large plaques, we studied both types of plaques. In small plaques, we found that all three factors co-localized in cells that were of macrophage appearance but not all spindle-shaped cells expressed all three factors (dashed circles in Fig. 2). In addition, the media beneath plaques also showed a lack of uniformity for all three factors, but ACE2-positive cells and separate Oct-4-positive cells were clearly visible (Fig. 2). In larger sized atherosclerotic plaques, all three factors co-localized in cells that were of macrophage appearance, similar to the situation in smaller plaques, and it was difficult to determine spindle-shaped cells owing to the large number of cells of macrophage appearance (Fig. 3). Sections of abdominal aorta from both animals killed at 4 weeks and animals killed at 14 weeks all showed negative staining to the negative control antibody. The present study was undertaken after we observed that disease was present at 4 weeks in our pilot study (4 week group shown with adjacent ACE2 immunoreactivity; Fig. 4).

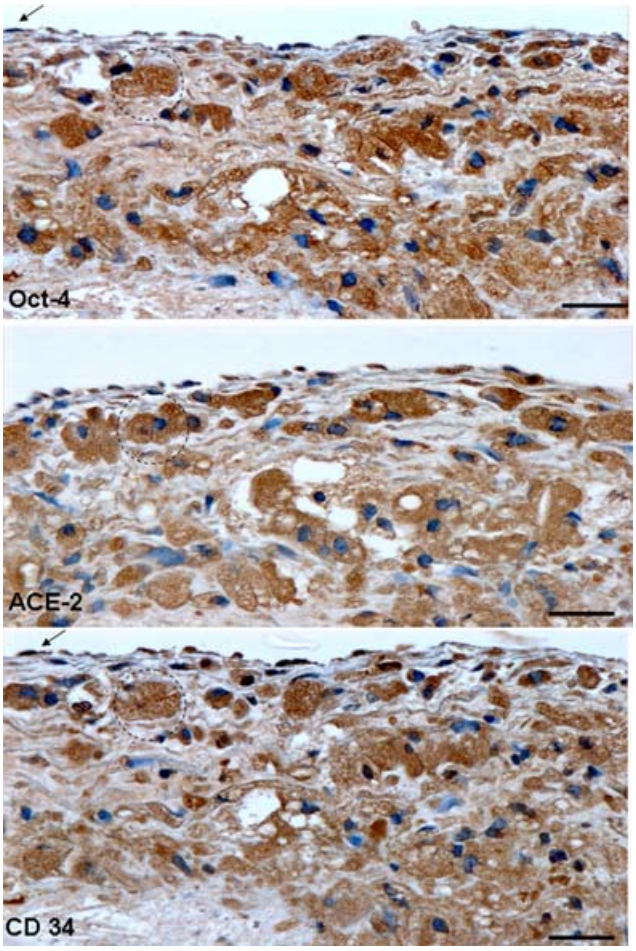

Figure 3. Photomicrographs of serial adjacent sections of rabbit abdominal aorta demonstrating Oct-4, ACE2 and CD34 immunoreactivity in large atherosclerotic plaques
Octomer-4, ACE2 and CD34 immunoreactivity was not as readily detectable in endothelium overlying these plaques; however, some specific cells were positive for ACE2, Oct-4 or CD34. Arrows point to an endothelial cell negative for Oct-4 but positive for CD34. Scale bars represent 25 μm.

Discussion

The results of the present study confirm the presence of cells within rabbit atherosclerotic plaques that co-immunolocalize ACE2 and Oct-4, as well as the haematopoietic stem-cell marker CD34 in both small and larger plaques. In our previous studies, we showed that macrophages and smooth muscle cells of rabbit atherosclerotic plaques expressed the haematopoietic

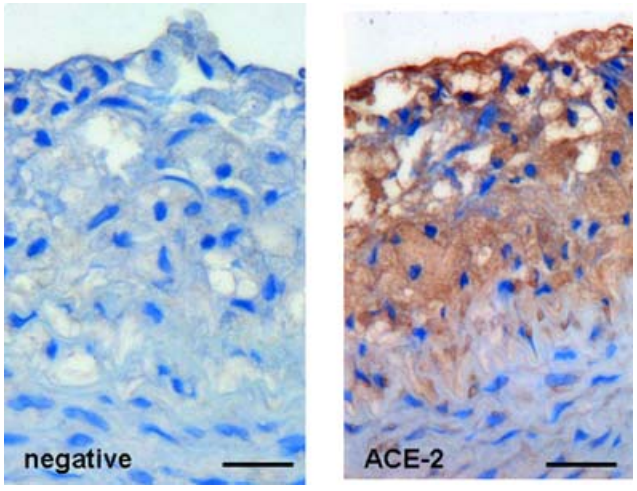

Figure 4. Photomicrographs of serial adjacent sections of rabbit abdominal aorta demonstrating negative control and ACE2 immunoreactivity in lipid-laden macrophages at the 4 week time point
At this time point, disease consists of lipid-laden macrophages binding to the surface of the blood vessel wall. Scale bars represent 25 μm.

stem-cell marker CD34, indicating that these cell could be of primitive origin (Zulli *et al.* 2005). We also showed that the RAS anti-atherosclerotic factors, ACE2 and the angiotensin II type 2 receptor, co-localize in rabbit atherosclerotic plaques (Zulli *et al.* 2006) and that such cells also express markers of embryonic stem cells, such as Oct-4 (Zulli *et al.* 2007*b*). In addition, our most recent publications clearly demonstrate that human diseased vessels also express markers of embryonic stem cells (Zulli *et al.* 2007*c*) and the RAS (Zulli *et al.* 2007*a*), indicating that cells in diseased arteries do express markers of primitive origin or might be primitive cells that have migrated into diseased tissues.

The presence of ACE2/Oct-4-positive cells within atherosclerotic plaques indicates that ACE2 could also be involved in the determination of cellular differentiation state. For example, a recent study of patients with severe acute respiratory syndrome (SARS) demonstrated that ACE2 expression correlated positively with the differentiation state of human airway epithelia. The authors show that infection of human airway epithelia by SARS coronavirus correlates with the state of cell differentiation and ACE2 expression and localization (Jia *et al.* 2005). In addition, ACE2 has been shown to be sharply upregulated in maturing oocytes, specifically at the time of oocyte maturation (Bobe *et al.* 2006). Thus, whether ACE2 expression correlates with Oct-4 expression or macrophage/smooth muscle cell/endothelial cell differentiation warrants further study. Indeed, retinoic acid is a compound that could be used both to upregulate ACE2 and to downregulate Oct-4.

Retinoic acid has also been shown in a variety of cell culture experiments to impact a variety of cellular processes. For example, treatment of embryonal carcinoma cells with retinoic acid was associated with profound changes in cell morphology that also expressed smooth muscle α-actin that also functionally responded to angiotensin II (Blank *et al.* 1995), indicating that other effects of retinoic acid might be to stimulate a more pro-constrictive cellular environment as well as ACE2. All-*trans* retinoic acid is also a potent stimulator of embryonic stem-cell differentiation, and it is suggested that one of the mechanisms of action is via decreasing Oct-4 (Faherty *et al.* 2005), which is a transcription factor whose expression is associated with an undifferentiated cell phenotype and is downregulated when the cells differentiate (Pesce & Scholer, 2001). For example, a recent study by Faherty *et al.* (2005) clearly shows that that the level of at-RA is an important determinant of cell fate. They showed that in embryonic stem cells, lower levels of retinoic acid (0.1–10 nM) promoted the formation of epithelial-like cells, whereas higher levels (100–10 000 nM) favoured differentiation into fibroblastic-like cells. This study suggests that the dose of at-RA is important in the determination of cell type; therefore, further experiments aimed at determining the dose of at-RA that allows for a more stable plaque phenotype are warranted.

In conclusion, we show clear evidence that cells within atherosclerotic tissues co-express ACE2 and Oct-4, as well as the haematopoietic stem-cell marker CD34. Since at-RA can upregulate ACE2 and downregulate Oct-4, we suggest that further studies into cardiovascular disease should be aimed at determining the role of at-RA in both blood pressure regulation and cell differentiation.

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