A growing body of evidence suggests that the angiotensin II fragments, Ang(1-7) and Ang(3-8), have a vasoactive role, however ACE2, the enzyme that produces Ang(1-7), or AT4R, the receptor that binds Ang (3-8), have yet been simultaneously localised in both normal and diseased human conduit blood vessels. We sought to determine the immunohistochemical distribution of ACE2 and the AT4R in human internal mammary and radial arteries from patients undergoing coronary artery bypass surgery. We found that ACE2 positive cells were abundant in both normal and diseased vessels, being present in neo-intima and in media. ACE2 positive immunoreactivity was not present in the endothelial layer of the conduit vessels, but was clearly evident in small newly formed angiogenic vessels as well as the vaso vasorum. Endothelial AT4R immunoreactivity were rarely observed in either normal and diseased arteries, but AT4R positive cells were observed adjacent to the internal elastic lamina in the internal mammary artery, in the neo-intima of radial arteries, as well as in the media of both internal mammary artery and radial artery. AT4R was abundant in vaso vasorum and within small angiogenic vessels. Both AT4R and ACE2 co-localised with smooth muscle cell α actin. This study identifies smooth muscle cell α actin positive ACE2 and AT4R in human blood vessels as well as in angiogenic vessels, indicating a possible role for these enzymes in pathological disease.

Key words: human blood vessels, AT4 receptors, ACE2, IRAP.

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ACE2 and AT4R have never been demonstrated in the human conduit vessels. Thus in this study, we sought to determine the immunolocalization of both proteins in diseased (radial arteries) and non-diseased (internal mammary arteries) human blood vessels.

Materials and Methods

Segments of human blood vessels (n=5 radial and n=5 internal mammary arteries) were obtained from patients undergoing coronary artery bypass grafting. Patients with a mean age of 64 years gave informed consent and all were receiving HMG CoA reductase inhibitors and antihypertensive treatment (ACE inhibitors or diuretics or beta adreno-receptor blockers). Only non-traumatized vessel segment were used in this study. This study was approved by the Austin Hospital Medical Research Ethics Committee and followed institutional guidelines. Harvesting was performed using techniques previously established in our operating theatres (Ruengsakulrach et al. 1999). All vessels were fixed in formalin and were then processed and embedded in a single paraffin blocks as previously described (Zulli et al. 2006b).

Immunohistochemistry

Sections were randomly selected, dewaxed, rehydrated and placed in 10 mM TrisCl, pH 7.4. Sections were then preincubated with 1% goat serum in 10 mM TrisCl (pH 7.4) for twenty minutes before incubating with the primary antibody diluted in 1% goat serum in 10 mM TrisCl (pH7.4). Mouse monoclonal IgG, ACE2 (Cat# ALX 804-715, Alexis Biochemicals, diluted 1:150), rabbit polyclonal IgG, AT4R (Cat # IRAP11-A, Alpha Diagnostic, Texas, diluted 1:100), smooth muscle cell α actin monoclonal antibody (Cat #M0851, Dakocytomation, diluted 1:100) and mouse monoclonal IgG against bacterial glucose oxidase (Cat # X0931, Dakocytomation, USA, diluted 1:50) and rabbit polyclonal IgG (Cat# X0936, Dakocytomation, diluted 1:50) were used as negative controls. All antibodies were incubated overnight. Immunohistochemistry for both monoclonal and rabbit polyclonal were performed using the Envision commercially available kit following the manufacturers directions (Zulli et al. 2005; Zulli et al. 2006b; Zulli et al. 2007). All antigenic sites were developed with DAB, counterstained with hematoxylin, dehydrated and mounted with DPX mounting media.

Results

ACE2 immunoreactivity was present throughout the vessel wall in all blood vessel types, albeit at differing intensities (Figure 1 F–J). Interestingly, ACE2 immunoreactivity was not observed in the endothelial layer of internal mammary arteries or radial arteries, but was clearly present in the medial layer of these vessels, especially apparent in the radial artery media (I, J) and rarely abundant in the IMA media (G). As well, ACE2 positive cells were present within the neo-intima of radial arteries (D, E). These cells co-localised with smooth muscle cell α actin present just beneath the internal elastic lamina (K, L). ACE2 was also clearly visible in both arterioles and venules of the vaso vasorum (venuels shown, M) and single positive cells in the adventitia were commonly observed (arrow, M). Negative control sections show no immunoreactivity (N, O).

AT4R immunoreactivity followed a similar pattern of staining to ACE2, being present throughout the vessel wall in all blood vessel types (Figure 2F–J). Like ACE2, AT4R immunoreactivity was not observed in the endothelial layer of internal mammary arteries or radial arteries, but was clearly present in the medial layer of these vessels, especially apparent in the radial artery media (I, J). As well, AT4R positive cells were present within the neo-intima of radial arteries (D, E). These cells co-localised with smooth muscle cell α actin present in the radial arteries (N, O). AT4R was also clearly visible in both arterioles (top of L) and venules (bottom of L) of the vaso vasorum. Endothelial AT4R immunoreactivity was observed in the arteriole (L), but the majority of the staining was observed in the media of the vessels, and correlated with smooth muscle cell α actin (M). Negative control sections to the polyclonal antibody shows no immunoreactivity (IMA shown in H, C).

In the fully occluded radial artery (Figure 3),
Figure 1. Photomicrographs of ACE2 immunoreactivity in IMA and RA. ACE2 immunoreactivity was observed in the media of both normal (IMA) and diseased (RA) arteries. Specifically, single positive cells were observed just beneath the internal elastic lamina of the IMA (A, B) that also co-localised with smooth muscle cell α-actin in serial sections (K, L), however some IMA had no definitive positive cells (H, C). In the RA (I, J), ACE2 was clearly present in the cells within the intima and media, and this has been magnified (D, E), which also co-localise with smooth muscle cell α-actin (Figure 2N, O). ACE2 was also observed in the vaso vasorum and a single positive cell in the adventitia is clearly visible (arrow). Negative controls (monoclonal) is shown (N, and magnified in O). Original magnifications are all 400x in F-J, N. Scale bars: Figures 1 A-E, K, L = 9 µm; Figures 1 F-J = 70 µm; Figure 1 N = 35 µm; Figure 1 O = 3.5 µm.

Figure 2. Photomicrographs of AT4R/IRAP immunoreactivity in IMA and RA. AT4R immunoreactivity was observed in the media of both normal (IMA) and diseased (RA) arteries. Similar to ACE2, single positive cells were observed just beneath the internal elastic lamina of the IMA (F, G and magnified in A, B) and in the media (K) that also co-localised with smooth muscle cell α-actin in serial sections (Figure 1K, L), however some IMA had no definitive positive cells (not shown). In the RA (I, J), AT4R was clearly present in the cells within the intima and media, and this has been magnified (D, E), which also co-localise with smooth muscle cell α-actin (Figure N, O). AT4R was also observed in the vaso vasorum and this also correlated with smooth muscle cell α-actin (M). Negative controls (polyclonal) is shown (H, and magnified in C). Original magnifications are all 200x in F-J, N. Scale bars: Figures 2 A-E, K = 9 µm; Figures 2 F-J = 140 µm; Figures 2 L, N = 70 µm; Figure 2 M = 35 µm Figure 20 = 7 µm.
ACE2 and AT4R was clearly present in the media of the vessel, but ACE2 was present in media of the small angiogenic vessels present within the granulation tissue, and this correlated with smooth muscle cell α actin staining (C, G). However, AT4R did not show positive staining in the media of angiogenic vessels (F), but endothelial AT4R staining was clearly present.

**Discussion**

These results show that both ACE2 and AT4R are localized in a range of human blood vessels with varying intimal pathologies. Moreover, ACE2 and AT4R were not easily detectable in the endothelium of conduit vessels, but AT4R was clearly evident in the endothelium of the vaso vasorum and in angiogenic vessels.

The amount of ACE2 in tissues appears to be specific to the type of cardiovascular disease. For example, it is increased in human and rat myocardial infarction (Burrell et al. 2005) and rabbit atherosclerotic plaques (Zulli et al. 2006a), but decreased in diabetes (Tikellis et al. 2003), and hypertension (Crackower et al. 2002). However, current opinion suggests that in the vasculature, the atherogenic and mitogenic effects of Ang II are indirectly counterbalanced by ACE2. Firstly, ACE2 might have a vasoprotective role simply because it removes local Ang II from tissue. In this way, ACE2 acts as a vascular mop, whereby an increase in ACE2 to parallel an increase in ACE would help minimize the detrimental effects of Ang II. Secondly, as ACE2 produces Ang (1-7), Ang (1-7) can not only inhibit both AT1R (Roks et al. 1999) and ACE activity (Deddish et al. 1998), but also have specific effects through its novel receptor, the mas oncogene product (Mas) (Santos et al. 2003). Through Mas, Ang-(1–7) might stimulate nitric oxide synthase (NOS) and counteract the effects of Ang II. The effects of Ang-(1–7) may also involve cross-talk with the angiotensin type 2 receptor (AT2R), and the bradykinin type two receptor (BK2R) (Ferrario 1998). Taken together, this data and the data pre-
sented in this study suggest a possible role for ACE2 and angiotensin (1-7) in cell proliferation and vasodilation in human vessels. The primary functions related to AT4R include memory acquisition and retrieval, cerebral blood flow regulation, kidney function, angiogenesis and cardiac hypertrophy (de Gasparo et al. 2000). In larger blood vessels however, very little information exists regarding the function or localisation of AT4R. In this study, we found that endothelial AT4R (IRAP) was easily detectable in the vascular vasorum and in angiogenic vessels, but not in the endothelial layer of conduit vessels. This finding supports a previous study by Hall and colleagues, where they found that coronary microvascular endothelial cells bound radio labelled Ang IV (Hall et al. 1995). Furthermore, we observed high levels of AT4R in the media of diseased human blood vessels indicating a possible role in atherogenesis and within angiogenic vessels indicating a possible role in angiogenesis.

It is difficult to speculate at this stage the effect of patient characteristics on the expression of ACE2 and AT4R in the radial and internal mammary arteries due to the sample size. The patients in this study were all receiving statin therapy with an additional hypertensive treatment such as ACE inhibition or beta adrenoceptor blockade. Further studies aimed at collecting larger samples and correlating patient characteristics with AT4R and ACE2 are ongoing.

In conclusion, we report high immunoreactivity for ACE2 and AT4R proteins in the media of diseased blood vessels as well as in angiogenic vessels, indicating a possible role for these factors in blood vessel remodelling and therefore might be involved atherogenesis.

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