Restricted expression of the gap junctional protein connexin 43 in the arterial system of the rat

TAO HONG AND CARYL E. HILL

Division of Neuroscience, John Curtin School of Medical Research, Australian National University, Canberra, Australia

(Accepted 10 March 1998)

ABSTRACT

Connexin 43 (Cx43) has been reported to be expressed in vascular smooth muscle cells and endothelial cells. Evidence for possible variations in Cx43 distribution within different parts of the vascular system is limited. We have therefore investigated the expression of Cx43 in the endothelia and media of 11 vessels of different size and function in the rat, using immunofluorescence and confocal laser scanning microscopy. The results showed that punctate Cx43 staining was abundant in the endothelia and media of all of the 5 elastic arteries examined. In the media, the amount of Cx43 staining decreased as the size of the elastic arteries became smaller. In the 6 muscular arteries examined, 2 different patterns of Cx43 staining were observed. In the first type, Cx43 expression was high in the endothelium but virtually absent from the media. Mesenteric resistance, hepatic and tail arteries were examples. In the second type, Cx43 staining was absent from both the media and the endothelium. The coronary, basilar, and middle cerebral arteries showed this appearance. The results suggest that expression of Cx43 is largely restricted to elastic arteries in the arterial system of the rat. The lack of immunodetectable Cx43 from the media of all muscular arteries examined, and from the endothelia of some of these arteries, raises the possibility of significant differences in the form of expression of Cx43 in these vessels or the presence of other connexins.

Key words: Vasculature; endothelia; gap junctions; media.

INTRODUCTION

Gap junctions permit the passage of ions and small molecules between 2 neighbouring cells via aggregates of low resistance transmembrane channels. Each junctional channel arises from the apposition of 2 hemichannels or connexons, 1 in the membrane of each of the 2 adjacent cells. In turn, each connexon consists of 6 subunits comprising members of the connexin family (Bruzzone et al. 1996; Kumar & Gilular, 1996; Sosinsky, 1996). Studies in which different connexins have been expressed either singly or in combination in cultured cells and oocytes have demonstrated that functional gap junctional channels can form from homotypic associations and also from certain, but not all, heterotypic associations, although the selectivity of these latter channels may vary from the corresponding homotypic channels (Willecke & Haubrich, 1996).

Three of the 14 different mammalian connexin genes, which have been cloned and sequenced, have been identified in the walls of blood vessels. These are connexin 43 (Cx43), connexin 40 (Cx40) and connexin 37 (Cx37) (Larson et al. 1990; Lash et al. 1990; Beyer et al. 1992; Reed et al. 1993). From studies of dye transfer and electrical coupling, it has been suggested that gap junctions mediate cell to cell communication between endothelial cells, between smooth muscle cells and between endothelial cells and smooth muscle cells in the vascular wall (Beny & Gribi, 1989; Segal & Beny, 1992; Little et al. 1995b). The resulting intercellular movement of ions and small molecules may contribute to the synchronisation of spontaneous (Chaytor et al. 1997) or nerve-mediated vasomotor responses (Christ et al. 1996), as well as growth responses, particularly following injury (Polacek et al. 1997; Yeh et al. 1997b).

An overall view of the expression of the 3 connexins in blood vessels would suggest that all 3 are expressed in endothelial cells (Larson et al. 1990; Beyer et al.
Tissue preparation

Wistar-Kyoto rats of either sex (aged 5–7 wk), weighing 120–210 g, were anaesthetised with intra-peritoneal ketamine (44 mg/kg) and xylazine (8 mg/kg) according to guidelines of the Animal Experimentation Ethics Committee of the Australian National University. Rats were perfused through the left ventricle, first, with a sterile saline solution containing 0.1% sodium nitrite, 0.1% bovine serum albumin (BSA) and 0.25% heparin (1000 U/ml) and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.34). In some experiments, fixation was effected using 2% paraformaldehyde in 0.1 M phosphate buffer. Eleven different elastic and muscular arteries were sampled. Each artery was sampled from 2–4 different rats. The elastic arteries were the aortic arch, thoracic aorta, abdominal aorta, carotid artery and superior mesenteric artery, while the muscular arteries were the mesenteric resistance arteries supplying the ileum, the hepatic artery from the coeliac artery to the point of entry of its branches into the liver parenchyma, the caudal artery from the base of the tail, the basilar artery, middle cerebral artery and its branches and the coronary artery.

The arteries were dissected, after perfusion of the rats with the fixative, and washed in phosphate buffered saline (PBS). For the mesenteric arteries, the surrounding fat and adjacent veins were stripped away. Left ventricular tissue was also removed to provide a positive control as the presence of abundant Cx43 staining has been well established in this tissue (see Gourdie, 1995; Severs, 1995). All samples were immersed in 30% sucrose in PBS overnight at 4 °C, embedded in Cryo-M-Bed (Bright Instrument Company), and frozen under dry ice. Cryosections (10 µm) were cut and mounted on slides coated previously with either gelatin or silane. Where possible, both longitudinal and transverse sections were cut in order to provide different views of the endothelium and smooth muscle cells which were themselves arranged longitudinally and circumferentially respectively in the vessel walls.

Sections from 2 different arteries were mounted on each slide separated by a row of sections of the left ventricle. Sections were dried for 30 min over phosphorus pentoxide, fixed for a further 10 min with 4% paraformaldehyde to reduce the detachment of tissues from slides, washed in 0.1 M glycine in PB for 10 min and then in PB.

Immunohistochemistry

Two different anti-Cx43 antibodies were used: (1) affinity purified polyclonal anti-Cx43 raised in rabbits against a synthetic peptide corresponding to residues contained in the C-terminal cytoplasmic domain of rat Cx43 (1:250, Zymed, CA); (2) monoclonal mouse anti-Cx43 raised against residues 252–270 of the cytoplasmic domain of rat Cx43 (1:250, Chemicon, CA). Both antibodies were confirmed by the manufacturers to react only to Cx43 in Western blots.
having no cross-reactivity to other connexins. Rabbit antihuman von Willebrand Factor (VWF, Dako, Denmark) was used to label endothelial cells and hence define the area occupied by the endothelium.

Tissue sections were preincubated for 1 h with PBS containing 2% BSA, 0.3% Triton and 0.04% sodium azide, then with rabbit anti-Cx43 or mouse anti-Cx43 diluted in the same solution for 36 h at room temperature in a humidified chamber. After washes in PBS, samples were incubated with affinity-purified biotinylated goat antirabbit (1:200, Jackson Immunoresearch Laboratories Inc, PA, USA) or biotinylated horse antimouse (1:200, Vector, CA, USA) immunoglobulins respectively for 1 h, depending on the primary antibody used. Sections were washed in PBS, incubated in Texas Red conjugated to streptavidin (1:200, Amersham, UK) for another 1 h at room temperature, washed in PBS and mounted in buffered glycerol. In some experiments, sections were incubated in more concentrated antibody solutions in which the Triton concentration was tested at 0.1%, as well as 0.3%. Primary antibody incubations were also carried out for 24 h.

FITC-conjugated swine antirabbit immunoglobulin (1:40, Dako, Denmark) was used for detection of the endothelial marker. Incubation with the antibody against VWF was undertaken after the staining for Cx43 had been completed. Sections were washed in PBS and incubated with rabbit antihuman VWF (1:300) for 1 h at room temperature and then with FITC antirabbit immunoglobulin for another hour. As a control against cross reactivity between the fluorophores detecting the Cx43 and VWF respectively, since they were both made in rabbits, sections were incubated with rabbit anti-Cx43 as described above and then with FITC antirabbit immunoglobulin for 1 h.

To control against false positive staining, several controls were included in each experiment. These included (1) omission of the primary antibody, (2) omission of both primary and secondary antibodies, and (3) preincubation of the primary antibody with the corresponding peptide at a hundred fold excess overnight at 4 °C before incubation on the sections. Left ventricular muscle was used as a positive control for the presence of Cx43 staining.

Data acquisition and analysis

All preparations were observed using a fluorescence microscope (BH2-RFL, Olympus) fitted with appropriate filters for fluorescein (excitation filter IF-190 + EY455, dichroic mirror DM500, barrier filter O-515) and Texas Red (excitation filter IF-545 + BG-36, dichroic mirror DM580, barrier filter R-610). For quantification of staining in each artery, a horizontal series of images was taken at 1 μm intervals through the tissue section using a confocal laser scanning microscope (Leica TCS 4D). For tissues double labelled with Cx43 and VWF, the same series was scanned with filters appropriate to both Texas Red and FITC. Each individual series was then combined to produce a single image of Cx43 staining throughout the 10 μm section as seen with the filters for Texas Red and a single image of the VWF staining of the same area with filters for FITC. The elastic laminae within the walls of the vessels were visible under fluorescence illumination.

Images obtained from the confocal microscope were saved as TIF files and analysed using the Micro-Computer Image Device (MCID, Imaging Research Inc, Canada). As the Cx43 staining was punctate, it was possible to use the grain counting function and link the 2 channels containing the Cx43 series image and the corresponding VWF series image. The area occupied by the endothelium, as seen with the VWF staining, could then be outlined in one channel and counts made of the Cx43 staining in the corresponding area in the second channel. Similarly, counts of Cx43 staining could be made in the media. Finally, by using the delineations of the elastic laminae, it was possible to further subdivide the media in the elastic arteries into a series of muscle layers. In the media and in the endothelium, data were obtained regarding the mean diameter of immunolabelled Cx43 plaques and the number of these plaques per unit area for each 10 μm thick image series or the number of immunolabelled Cx43 plaques per 10 μm². Thus comparisons could be made between different tissue layers within a single vessel and among the 11 different vessels. By expressing data as plaques per standard volume, account could be taken of the varying size of the different vessels. For each vessel, data were obtained from 2–4 rats. In turn, the data for each rat were averaged for analysis of at least 3 confocal images.

Statistical analysis on the data was made using a 1-way analysis of variance (ANOVA) followed by individual pairwise t tests with Bonferroni correction for multiple group comparisons. Significance was assessed at the 5% level.

RESULTS

General observations

Because the cells of the endothelium and media are oriented perpendicular to each other, tangential,
transverse and longitudinal sections presented different structural views of the arterial wall. Transverse and tangential sections allowed better viewing of the staining pattern of the endothelial cells, whilst longitudinal sections showed more of the smooth muscle cells which are oriented perpendicular to the longitudinal axis of the vessels. Consequently, vessels were aligned and cut longitudinally to maximise the contact area between adjacent smooth muscle cells. In tissue prepared in this way, tangential sections of endothelia were often found.

In order to safeguard against false negatives, a positive control of ventricular muscle was present on all slides. In all cases the myocardial tissue exhibited punctate staining in the intercalated disks and along the lateral surfaces of neighbouring myocytes (Fig. 1a, arrows). No staining was observed if ventricular tissue was incubated sequentially with rabbit anti-Cx43 (1:250) and then FITC-antirabbit immunoglobulin, indicating that the concentration of the Cx43 antibody was below the limit of detection when only a single secondary antibody was used. This is in contrast to the intense staining following sequential incubation in rabbit anti-Cx43 (1:250), biotinylated antirabbit immunoglobulin and streptavidin-Texas Red as described above.

Staining patterns in the media and endothelium of the various arteries were the same, irrespective of which primary antibody was used. The specificity of antibodies against Cx43 was demonstrated by the lack of staining when the tissue was incubated without the primary antibody or secondary antibodies, or when
the antibody was preincubated with the appropriate antigen (Fig. 1b). In our hands, however, some nonspecific punctate staining was found in the internal elastic lamina of muscular arteries when using the monoclonal antibody. Consequently, all analyses were performed on sections stained with the rabbit polyclonal antibody.

**Cx43 expression in elastic arteries**

Punctate Cx43 staining was abundant in the endothelium and media of all the elastic arteries examined (Figs 2–4). Immunoreactive plaques in the endothelium were aligned parallel to the axis of the vessels, consistent with the orientation of endothelial cells (Figs 3a, 4a). In the media, staining was arranged circumferentially around the vessel wall mimicking the orientation of the smooth muscle cells (Fig. 3c). This phenomenon was particularly obvious in tangential sections (Fig. 4a). In many sections, however, it was difficult to distinguish the staining in the endothelium from that in the media (see Fig. 2a) and hence double labelling with the antibody against VWF was routinely performed in order to identify the endothelial layer (see Fig. 2b).

The media of elastic arteries was seen to be divided into layers by the presence of multiple sheets of elastic laminae (Figs 2b, 3a). In the media, most of the Cx43 labelling could be seen within these smooth muscle layers and in general the staining was more intense in the middle and outer layers (Figs 3a, 4a). Some staining was also observed in the elastic laminae (Figs 2a, 3a).

The amount of Cx43 staining in the media decreased as the size of the elastic arteries became smaller. Thus the aortic arch showed the most abundant Cx43 expression in the media, followed by the thoracic aorta and carotid arteries. The superior mesenteric artery and abdominal aorta showed the least staining with anti-Cx43. Immunolabelled gap-junctional plaques in these vessels were sparse and less intense. On the other hand, Cx43 staining in the endothelium was universally dense and intense amongst these various arteries.

**Cx43 expression in muscular arteries**

Two different patterns of Cx43 staining were observed in muscular arteries. In the first type, Cx43 expression was high in the endothelium but absent in the media. Mesenteric resistance arteries, hepatic and tail arteries were examples, exhibiting abundant staining along
Fig. 4. Composite confocal image of a 10 µm section from the carotid artery (a) and a side branch (b, c). In a, abundant Cx43 staining can be seen in the endothelium (E) and the media (M). Note the perpendicular orientation of the staining in the 2 layers. In the media, more Cx43 staining can be seen in the middle and outer layers. In b and c, a side branch of the carotid artery is stained with antibodies against Cx43 (b) and VWF (c) to demonstrate the endothelial layer. Cx43 staining is present in the endothelium (E) but not in the media (M) of the side branch. The outer edge of the media is delineated by arrowheads in b and c. The internal elastic lamina can be seen in grey in c. Bar, 50 µm.

Fig. 5. Composite confocal image of a 10 µm section of a mesenteric resistance artery stained with either antibodies against Cx43 (a) or VWF (b). Cx43 staining is present along the margins of the endothelial cells (E), but not in the media (M) in a. The outer edge of the media is delineated by arrowheads in a and b. In b, the endothelial cells are stained with the antibody against the VWF. The internal elastic lamina is visible in grey. Bar, 50 µm.

The membranes of adjacent endothelial cells but little or no staining in the media (Fig. 5). This pattern of staining was also seen at the points where muscular arteries branched from parent elastic arteries (Figs 3c, 4b, c). In these areas, Cx43 expression was sharply decreased in the media, although the staining in the endothelium persisted (Figs 3c, 4b, c).

In the second type of muscular artery, Cx43 staining was absent from both the media and the endothelia. The coronary, basilar, and middle cerebral arteries showed this appearance (Fig. 6). The lack of staining was not due to methodological problems as a similar appearance was found even when different fixation conditions, incubation solutions and antibody con-
Fig. 6. Composite confocal image of a 10 µm section of basilar (a), middle cerebral (b, d) and coronary artery (c). In the basilar artery, there is little evidence for Cx43 staining in the endothelium (E) or in the media (M). The inner edge of the endothelium (E) is delineated by arrowheads. Abundant staining of Cx43 can be seen in the pia (arrowheads plus asterisk) which surrounds the vessel. In b and d, the middle cerebral artery is stained with antibodies against Cx43 (b) and VWF (d). Note that there is little evidence of staining in either the endothelium (E) or the media (M). The inner edge of the endothelium (E) is delineated by arrows. Staining can be seen in the surrounding pia (arrowheads plus asterisk). The coronary artery (CA) shows no Cx43 staining in either the endothelium or media. Abundant Cx43 staining can be seen in the surrounding ventricular muscle. Bar, 50 µm.

Quantification of Cx43 expression

In order to accurately define the endothelial and medial areas for quantifying the Cx43 immunolabelling, double labelling using the endothelium marker was routinely performed. Figures 2b, 3b, 4c, 5b and 6d demonstrate clearly that VWF staining was confined to the endothelium and could therefore provide a template for the computer analysis of staining density in the endothelium and also the media.

Quantification of the Cx43 staining using the grain counting function of the MCID program as described in the methods, confirmed the observations above (Tables 1, 2). Essentially 3 different staining patterns were observed. Elastic arteries, such as the aortic arch, thoracic aorta, carotid artery, superior mesenteric...
Table 1. Average diameter and density of Cx43 plaques in endothelial cells of different arteries

<table>
<thead>
<tr>
<th>Artery</th>
<th>Diameter of plaque (μm)</th>
<th>Density (plaques per 10 μm²)</th>
<th>Number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic arch</td>
<td>0.556 ± 0.014*</td>
<td>0.119 ± 0.055</td>
<td>3</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>0.519 ± 0.007</td>
<td>0.163 ± 0.079</td>
<td>2</td>
</tr>
<tr>
<td>Carotid artery</td>
<td>0.476 ± 0.057</td>
<td>0.078 ± 0.010</td>
<td>4</td>
</tr>
<tr>
<td>Superior mesenteric artery</td>
<td>0.574 ± 0.015</td>
<td>0.091 ± 0.014</td>
<td>3</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>0.525 ± 0.014</td>
<td>0.092 ± 0.064</td>
<td>4</td>
</tr>
<tr>
<td>Hepatic artery</td>
<td>0.418 ± 0.067</td>
<td>0.159 ± 0.027</td>
<td>3</td>
</tr>
<tr>
<td>Tail artery</td>
<td>0.460 ± 0.059</td>
<td>0.072 ± 0.024</td>
<td>3</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>0.423 ± 0.110</td>
<td>0.073 ± 0.027</td>
<td>3</td>
</tr>
<tr>
<td>Basilar artery</td>
<td>0.505 ± 0.020</td>
<td>0.00442 ± 0.00159</td>
<td>3</td>
</tr>
<tr>
<td>Middle cerebral artery</td>
<td>0.506 ± 0.054</td>
<td>0.00744 ± 0.00472</td>
<td>3</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>0.566 ± 0.034</td>
<td>0.0064 ± 0.00254</td>
<td>3</td>
</tr>
</tbody>
</table>

* Values are means and standard errors of means

Table 2. Average diameter and density of Cx43 plaques in smooth muscle cells of different arteries

<table>
<thead>
<tr>
<th>Artery</th>
<th>Diameter of plaque (μm)</th>
<th>Density (plaques per 10 μm²)</th>
<th>Number of rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic arch</td>
<td>0.557 ± 0.012*</td>
<td>0.186 ± 0.020</td>
<td>3</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>0.549 ± 0.010</td>
<td>0.100 ± 0.042</td>
<td>2</td>
</tr>
<tr>
<td>Carotid artery</td>
<td>0.485 ± 0.024</td>
<td>0.125 ± 0.038</td>
<td>4</td>
</tr>
<tr>
<td>Superior mesenteric artery</td>
<td>0.524 ± 0.019</td>
<td>0.036 ± 0.022</td>
<td>4</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>0.544 ± 0.027</td>
<td>0.015 ± 0.006</td>
<td>4</td>
</tr>
<tr>
<td>Hepatic artery</td>
<td>0.334 ± 0.038</td>
<td>0.004 ± 0.001</td>
<td>3</td>
</tr>
<tr>
<td>Tail artery</td>
<td>0.421 ± 0.108</td>
<td>0.001 ± 0.0007</td>
<td>2</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>0.376 ± 0.190</td>
<td>0.0001 ± 0.0001</td>
<td>2</td>
</tr>
<tr>
<td>Basilar artery</td>
<td>0.492 ± 0.008</td>
<td>0.001 ± 0.0002</td>
<td>3</td>
</tr>
<tr>
<td>Middle cerebral artery</td>
<td>0.544 ± 0.042</td>
<td>0.001 ± 0.0009</td>
<td>3</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* Values are means and standard errors of means

artery and abdominal aorta, showed staining in the endothelium and in the media. While the staining in the endothelium was uniformly dense amongst the arteries, the superior mesenteric artery and abdominal aorta showed less medial staining than the other 3 elastic arteries examined (Tables 1, 2). Muscular arteries, on the other hand, showed staining in the endothelium but not in the media (hepatic, tail and mesenteric resistance arteries) or no staining in either the endothelium or the media (basilar, middle cerebral and coronary arteries; Tables 1, 2).

Statistical analysis in general confirmed these subdivisions. One way ANOVA showed no significant variation amongst the densities of Cx43 staining in the endothelia amongst the various arteries (P = 0.066). However, if the arteries were grouped as elastic and muscular with average densities of 0.133 ± 0.0336 (n = 16) and 0.0538 ± 0.0149 (n = 18) respectively, there was a significant difference (P = 0.032) between the staining in the endothelia of the 2 groups. Further subdivision of the muscular arteries for statistical analysis was considered to be too subjective. In the media (Table 2), ANOVA showed a significant variation in the density of staining amongst the arteries (P < 0.0001). Pairwise comparisons with Bonferroni correction confirmed the lack of significant difference in the staining amongst the aortic arch, thoracic aorta and carotid artery (P > 0.05) and the presence of a significant difference between the staining in these arteries and the remaining ones (P < 0.05).

The average diameter of the immunolabelled Cx43 plaques in both the endothelium and smooth muscle cells is also presented in Tables 1 and 2. In general, plaques were between 0.3 and 0.5 μm in diameter in both the endothelium and in the media of all vessels. In multiple group comparisons, no statistical differences existed between the average diameter of the immunolabelled Cx43 plaques either in the same tissue layer between vessels or between the endothelium and the media of the same vessel.

In all the analyses using the MCID program, care was taken to ensure that the setting of criteria to distinguish positive staining above background ac-
curately represented the staining in the original images. Thus the target colour was set to just cover the Cx43 plaques in the original images. Where artefacts appeared or appropriate staining was eliminated through these processes, it was possible to erase or add the plaques back respectively, after comparison with the original images. Validation of this procedure has been reported elsewhere (Green et al. 1993; Blackburn et al. 1997). In line with these comments, higher nonspecific background in sections of the hepatic artery may have artificially led to the apparently smaller diameter of the plaques in this vessel compared with other vessels. The relative size of plaques between the endothelium and media would not, however, be affected by the setting of background criteria.

DISCUSSION

In the present study using immunohistochemistry, we have demonstrated that Cx43 is not uniformly distributed throughout the arterial system of the rat. In elastic arteries, expression is high in both the endothelium and smooth muscle of the media. The most intense staining was found in the media of the larger vessels near the heart. On the other hand, expression in the media of muscular arteries was virtually absent. Muscular arteries could be further divided into those arteries which showed Cx43 expression in the endothelial cells and those in which Cx43 expression appeared to be entirely lacking from both endothelial cells and smooth muscle cells. Arteries of the latter type appeared prominent in the brain and in the heart. The most striking examples of the difference between expression in elastic and muscular arteries could be seen at the branch points where muscular arteries arose from elastic arteries. The dense circumferential distribution of Cx43 plaques at the origin of the muscular artery was rapidly lost while expression was continuous within the endothelium. A similar observation has been made for the zone close to the junction of the coronary artery with the aorta in the rat: the aorta showed abundant Cx43 staining in both the endothelium and the media while the coronary artery did not show any Cx43 staining in either tissue layer (Yeh et al. 1997a).

In elastic arteries, Cx43 expression could be clearly identified in both the endothelium and the media. Several previous studies have reported a similar pattern of expression in the aorta in vivo (Larson et al. 1990; Reed et al. 1993; Polacek et al. 1997; Yeh et al. 1997a) and in aortic endothelial and smooth muscle cells in culture (Larson et al. 1990; Lash et al. 1990; Beyer et al. 1992; Pepper et al. 1992; Moore & Burt, 1995). In contrast, Bruzzone et al. (1993) reported Cx43 expression only in the media but not in the endothelium of the aorta. In the present study, we used a double labelling technique to identify the region occupied by the endothelial cells and this could clearly be seen to contain specific staining for Cx43. Yeh et al. (1997a), using a similar technique, also demonstrated staining in the endothelium of the aorta and pointed out that some confusion can arise due to the detachment of endothelial cells during tissue preparation. While messenger RNA for Cx43 has been reported to be expressed in the endothelia and media of the rabbit iliac artery, another elastic artery (Polacek et al. 1997) and recently, in the rabbit superior mesenteric artery (Chaytor et al. 1997) and rat carotid artery (Yeh et al. 1997b), no other elastic arteries have been studied to date. In the present study, we have shown Cx43 expression in a number of elastic arteries which include the aortic arch, carotid artery, superior mesenteric artery, thoracic and abdominal aorta. Taken together, the results suggest that Cx43 may be expressed in all elastic arteries although the level of expression in the media may decrease with increasing distance from the heart.

Within the walls of elastic arteries, Cx43 expression was more intense in the middle and outer layers of smooth muscle cells. This may reflect that there are a greater number of smooth muscle cells in these outer laminae or simply that there are more gap junctions per cell. Yeh et al. (1997b) have reported a similar gradient in Cx43 gap junctions in the media of the rat carotid artery and have further demonstrated an increase in gap junctional area per cell. It is interesting that the nerves which control these vessels are confined to the medial-adventitial border and coordination of nerve-mediated responses would require efficient cell coupling. Staining was also observed within the elastic laminae which separated the various layers of smooth muscle cells. This is consistent with the appearance of occasional cellular cross bridges between the muscular units (Blackburn et al. 1997) and would facilitate layer to layer communication through the elastic laminae.

In contrast to the expression of Cx43 in the media of elastic arteries, there was little evidence for expression in the media of muscular arteries. On the other hand, Cx43 was present in the endothelium of some muscular arteries, such as the hepatic, tail and mesenteric resistance arteries, but absent from both tissue layers in other arteries, such as the basilar, middle cerebral and coronary. The lack of staining of
the blood vessels in these tissues was accompanied by abundant specific Cx43 staining in the epithelial cells of the pia and cardiac myocytes. Few muscular arteries have been investigated previously. Our results are in line with the lack of Cx43 staining in the media of the coronary artery in a number of species (Bastide et al. 1993; Bruzzone et al. 1993; Mikkelsen et al. 1993; Gros et al. 1994; Verheule et al. 1997; Yeh et al. 1997a). In the endothelium of the coronary artery, Cx43 has also been reported to be absent (Bruzzone et al. 1993; Mikkelsen et al. 1993; Gros et al. 1994; Verheule et al. 1997; Yeh et al. 1997a) or only weakly expressed (Bastide et al. 1993). Some evidence for species variation existed in that Cx43 was reported to be present in the media, but not the endothelium, of the coronary artery in humans (Yeh et al. 1997a). In contrast to our findings of a lack of Cx43 in intracranial vessels, Little et al. (1995a) reported the presence of Cx43 and Cx40 in both the endothelium and media of arterioles in the pia. Since these authors also reported staining in arterioles in the cremaster muscles of the rat and in the cheek pouch of guinea pig, it is possible that further variations in connexin distribution may occur in the smallest arterioles.

The mean diameter of immunolabelled Cx43 plaques was between 0.3 and 0.5 µm, irrespective of whether the plaques were located in the endothelium or in the smooth muscle cells. These values may be slightly overestimated due to the possible merging of connexin plaques throughout the 10 µm thick section, especially in vessels with dense connexin staining. Interestingly, the size of Cx43 gap junctional plaques in the rat aorta has also been reported to be similar between smooth muscle cells and between endothelial cells, although the density of Cx43 immunogold labelling within each plaque at the ultrastructural level was lower for plaques between endothelial cells than for those between smooth muscle cells (Christ et al. 1996). In contrast, Little et al. (1995a) reported an average size of immunolabelled plaques in the media of pial arterioles similar to those of the present study, but significantly larger plaques in the endothelial cells. The reason for this discrepancy is unlikely to arise from the use of the computer software for the analysis. Any slight change in size of the plaques as a result of the setting of criteria in the computer program would affect plaques in the endothelium and media equally and maintain, or even enhance, a size differential. Furthermore, Green et al. (1993) concluded that the size of gap junction plaques was similar whether the data were accumulated directly from freeze-fracture electron microscopy and or from automatic computer analysis of confocal images.

The results of the present study point to the general absence of Cx43 in the media of muscular arteries and in the endothelium of some of these vessels. Cx40 is the only other connexin reported to be present in vascular smooth muscle cells. It has been reported to be expressed in vascular smooth muscle cells in culture (Beyer et al. 1992; Moore & Burt, 1995), although it should be borne in mind that cultured cells may show changes in the expression of cellular proteins compared to the situation in vivo (Stutenkemper et al. 1992; Willecke & Haubrich, 1996). Indeed, the evidence in vivo would suggest that the expression of Cx40 is rare in the media of muscular and elastic arteries. It is present in the media of pial arterioles (Little et al. 1995a), but absent from the media of the coronary artery (Bastide et al. 1993; Bruzzone et al. 1993; Verheule et al. 1997; Yeh et al. 1997a), pulmonary artery (Bruzzone et al. 1993) and aorta (Bruzzone et al. 1993; Yeh et al. 1997a). Preliminary studies in our own laboratory using antibodies against Cx37 and Cx40 in the elastic and muscular arteries studied here have shown that expression of these 2 connexins is restricted to endothelial cells. It is unlikely that the present results reflect the absence of any connexin in muscular arteries since previous studies have provided evidence for the presence of significant electrical and chemical coupling in resistance vessels (Hirst & Edwards, 1989; Beny & Connat, 1992; Segal & Beny, 1992; Beny & Pacicca, 1994; Little et al. 1995b). It is possible, however, that the absence of immunohistochemical staining at the light microscope level may indicate that connexin plaques in these vessels are very small and below the limit of detection. Further studies of mRNA expression and immunohistochemistry at the ultrastructural level should resolve these issues. Alternatively, the present results may point to the presence of additional, as yet unidentified, members of the connexin family in the media and endothelia of many resistance vessels.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the World Health Organization. T. H. is grateful for the support of a Fellowship from the World Health Organization.

REFERENCES

Restricted Cx43 expression in rat arteries


