Cell and Tissue Research

DOI 10.1007/s00441-002-0623-y

Regular Article

Kit-like immunopositive cells in sheep mesenteric lymphatic vessels

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Received: 29 April 2002 / **Accepted:** 23 July 2002 / **Published online:**

Abstract. Recent electrophysiological studies have suggested that there is a subpopulation of cells in lymphatic vessels which act as pacemakers controlling the characteristic spontaneous contractile activity in this tissue. In this study, electron microscopy and immunohistochemical techniques were used on sheep mesenteric lymphatic vessels to investigate the morphology of the cells comprising the lymphatic wall. The smooth muscle cells were not orientated in circular and longitudinal layers as is seen in the gastrointestinal tract, but were arranged in bundles which interlock and cross over in a basket-weave fashion. Antibodies to Kit and vimentin, which are widely used to label specialised pacemaking cells in the gastrointestinal tract (known as interstitial cells of Cajal), demonstrated the existence of an axially orientated subpopulation of cells lying between the endothelium and the bulk of the smooth muscle. Examination of this area using electron microscopy showed cells which were electron dense compared to the underlying smooth muscle and contained caveolae, Golgi complexes, mitochondria, 10-nm filaments, a well-developed endoplasmic reticulum and a basal lamina. The smooth muscle cells typically contained caveolae, dense bodies, mitochondria, abundant filaments, sER and basal laminae. Cells dispersed for patch-clamp studies were also stained for vimentin and myosin. Myosin-staining cells had the typical spindle appearance of smooth muscle cells whereas the vimentin-positive cells could either be branched or more closely resemble the smooth muscle cells. The present study provides the first morphological evidence that specialised cells exist within the vascular system which have the ultrastructural characteristics of pacemaker cells in other tissues and are vimentin and Kit positive.

Keywords. Pacemaker - Smooth muscle - Kit - Vimentin

Introduction

Lymphatic vessels are spontaneously contractile and their regular phasic contractions are known to propel lymph in the living animal (Hall et al. *1965*) and in isolated preparations (McHale and Roddie *1976*). While it is now widely accepted that lymph is propelled mainly by the pumping activity of lymph ducts (Aukland and Reed *1993*) the origin of this spontaneous activity and its mode of propagation are poorly understood. Horstmann (*1959*) suggested that initiation of lymphatic contractions and their propagation are purely mechanical events. He argued that distension of a segment due to filling with lymph initiated a contraction which propelled lymph into the adjacent segment which dilated it and the process was repeated. This simple explanation, however intuitively appealing, cannot adequately account for the fact that isolated rings of sheep mesenteric lymphatic can contract regularly for many hours under conditions where there is no intraluminal distending force (Hollywood and McHale *1994*). This would suggest that there are pacemaker cells capable of generating a regular rhythm even when vessels are empty. It is still not clearly established whether spontaneous electrical activity is a property of all the smooth muscle cells in the lymphatic wall or of a subset of specialised pacemaking cells although recent evidence favours the latter view. Two currents which are thought to underlie pacemaking in the sinu-atrial node of the heart have recently been described in freshly isolated cells from sheep mesenteric lymphatic vessels. These are a hyperpolarisation-activated current I_f (McCloskey et al. *1999*) and a transient calcium current $I_{Ca(T)}$ (Hollywood et al. *1997*). These currents, however, were found in less than 5% of the cells studied and led us to suspect that there might be a distinct subpopulation of cells that were specialised for pacemaking. The purpose of the present study was to establish whether or not there was such a subset of cells that could be distinguished morphologically or immunohistochemically from the bulk of smooth muscle cells.

Materials and methods

Mesenteric lymphatics from sheep of either sex were obtained from a local abattoir immediately after slaughter. Vessels were either fixed immediately for electron microscopy (see below) or transferred to the laboratory in warm, oxygenated Krebs' solution. For whole-mount immunohistochemistry, tissues were pinned to the base of a Sylgard elastomer (Dow Corning) filled petri dish and surrounding fat and connective tissue were removed by sharp dissection. Where dispersed cells were used these were obtained by collagenase dispersal as described by McCloskey et al. (*1999*).

Cryostat methods

Lymphatic vessels were cannulated and distended by filling with either acetone (c-kit) or paraformaldehyde (vimentin). After fixation, 1-cm lengths of vessel were obtained by sharp dissection and washed overnight in phosphate-buffered saline (PBS). Tissues were cryoprotected for a total of 4 h in ascending concentrations of sucrose in PBS (1%, 5%, 10% and 20% w/v) before being embedded in moulds containing Lipshaw Embedding Matrix (Shandon Scientific) followed by rapid freezing in liquid nitrogen. Transverse sections (10 µm) were cut using a cryostat, collected on microscope slides and allowed to air dry before labelling with antibodies. Sections were blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature before addition of primary antibodies in 1% BSA and 0.1% Triton X-100. After 24 h incubation and several hours of washing in PBS, secondary fluorescent antibodies were added for 1 h. After washing in PBS, sections were viewed using a confocal microscope.

Immunohistochemistry

Lymphatic vessels were incubated in 1 µm nifedipine (Bayer) in Krebs' solution for 30 min and then stretched to approximately 150% of their resting length and width to show smooth muscle cells in their relaxed state. Vessels were fixed either in PBS-buffered paraformaldehyde (PFA) solution (4% w/v; 20 min, room temperature) or acetone (10 min, 4°C) followed by overnight washing in PBS at 4°C with several changes of solution. To reduce non-specific antibody binding, tissues were preincubated in 1% BSA solution for 1 h at room temperature. Tissues were incubated in primary antibody as follows: anti-smooth muscle myosin 1:100 (Sigma) for 24 h on PFA-fixed tissue, anti-vimentin 1:100 (Sigma) for 24 h on PFA-fixed tissue and anti-Kit 1:50 (Santa Cruz Biotechnology) for 36 h on acetone-fixed tissue. All antibodies were made up in PBS containing 1% BSA and 0.3% Triton X-100 (both from Sigma). Tissues were then washed for 24 h in PBS before incubation in secondary antibodies (diluted to 1:100 in PBS) for 1 h at room temperature. Anti-smooth muscle myosin was detected with anti-mouse FITC (Sigma), anti-vimentin and anti-Kit with anti-goat FITC (Sigma). F-actin staining was achieved by incubating the tissue in phalloidin-TRITC for 24 h (in 0.3% Triton X-100 and 1% BSA). All tissues were washed in PBS for several hours before mounting in buffered Gelvatol. Control tissues were prepared in a similar manner omitting the primary antibody from the incubation solution. Specimens were examined using a Nikon Diaphot microscope with brightfield and fluorescence microscopy using the appropriate filter combination for FITC (excitation at 488 nm) and also imaged using a Biorad MRC 600 confocal laser scanning microscope. Micrographs shown in this paper are digital composites of Z-series scans of 10-20 optical sections through a depth of 5-20 µm. Final images were constructed using Imaris (Bitplane AG) software.

Electron microscopy

Tissues were fixed by immersion in 3% glutaraldehyde in 0.1 M cacodylate buffer in the abattoir immediately after removal from the animal. To ensure adequate, rapid fixation and to maintain shape, vessels were also cannulated using a fine needle and filled with fixative. After 1 h, vessels were cut into smaller segments of approximately 5-10 mm in length and placed into fresh fixative for 4 h. After washing overnight in cacodylate buffer, tissues were postfixed in 0.1 M osmium tetroxide made up in 0.1 M cacodylate buffer, washed briefly in distilled water, dehydrated in a graded ethanol series and infiltrated with and embedded in TAAB embedding resin. Semithin sections were cut transversely and parallel to the long axis of the vessel and stained with toluidine blue. Ultrathin sections (70 nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate before viewing with a Jeol 100LXII electron microscope.

Results

Structure of the lymphatic wall

Lymphatic vessels were examined using electron microscopy to investigate the identity and ultrastructure of the cells making up the wall. Figure 1 shows a typical electron micrograph taken from a transverse section of a sheep lymphatic vessel which was distended before and during fixation. The lumen of the vessel was lined with a continuous sheet of endothelial cells similar to that reported in bovine (Hanley et al. *1992*) and in guinea-pig mesenteric lymphatic vessels (Crowe *1996*). Lying below this was a layer of electron-dense cells which appeared to form an inner ring around the circumference of the vessel. The greater part of the wall consisted of a layer, approximately ten cells thick, of smooth muscle cells. The cells were seen in longitudinal, oblique or transverse section and this rather unusual orientation was obtained whether the tissue was cut in a transverse or a longitudinal

plane of section. This suggested that lymphatic smooth muscle cells are not arranged in clear longitudinal and circular layers as in the gastrointestinal tract but may be made up of interlocking bundles of cells running in many different directions (see Fig. 3). An extensive array of collagen fibres was seen throughout the wall and especially on the adventitial surface (Fig. 1). Fibroblasts were seen in the collagen-filled spaces throughout the wall but were most abundant on the adventitial surface. These were easily distinguished from other cell types by their distinctive rough endoplasmic reticulum in the form of dilated cisternae. Blood capillaries forming the lymphatic's vasa vasorum were also observed in the adventitia and, less frequently, mast cells were encountered.

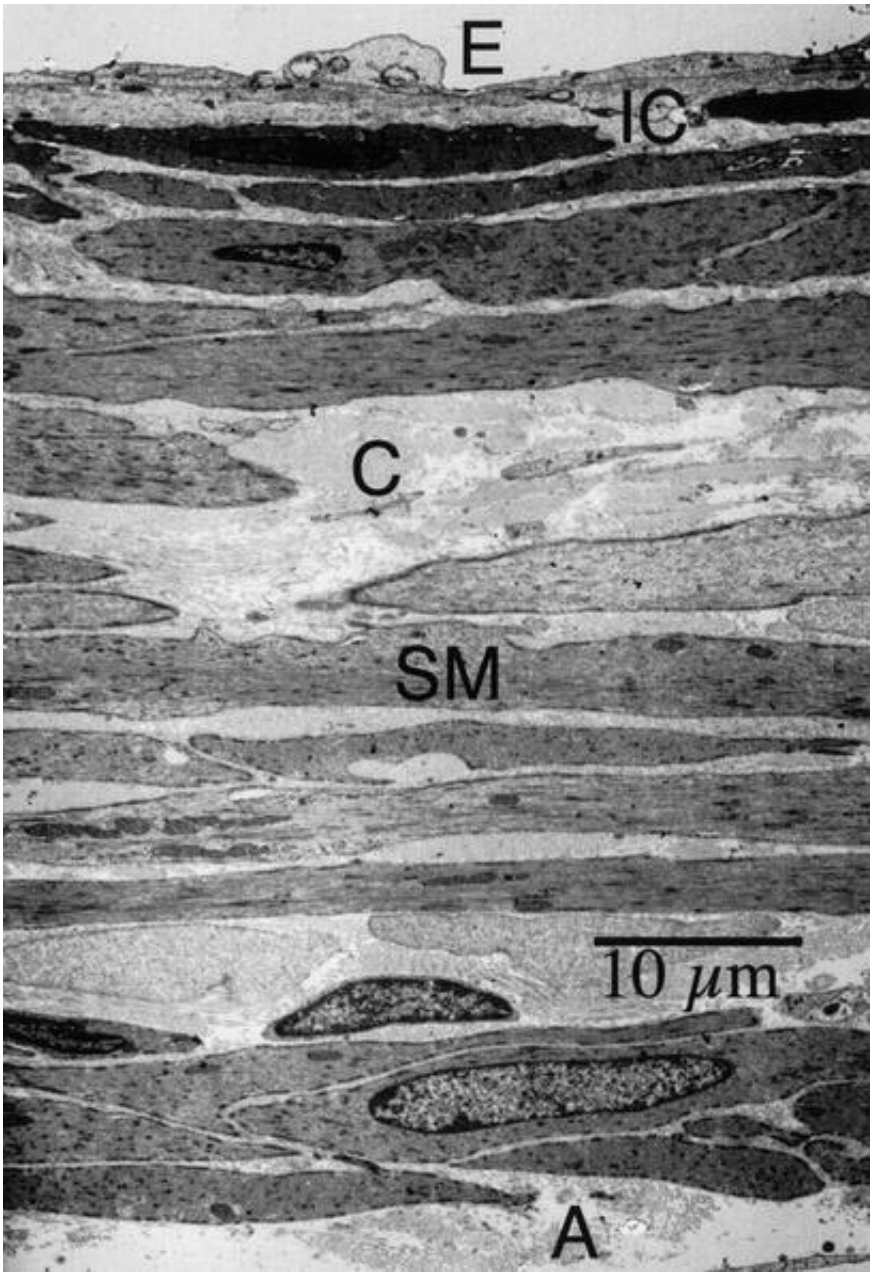

Fig. 1. Transverse section of the wall of a sheep mesenteric lymphatic. The lumen of the vessel was lined with endothelium (*E*) below which lay electron-dense cells or interstitial cells (*IC*). The majority of the cross-sectional area of the wall consisted of smooth muscle cells (*SM*) which were sectioned in many different orientations. Interspersed between smooth muscle bundles lay numerous collagen fibres (*C*) and these were particularly abundant in the adventitia (*A*). *Scale bar* represents 10 µm

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The electron-dense cells lying below the endothelium had many of the features described in interstitial cells of Cajal (ICC) seen in gastrointestinal preparations (for review see Rumessen and Thuneberg *1996*). Sections of these cells at higher magnification are shown in Fig. 2. Caveolae, mitochondria, smooth and rough endoplasmic reticulum and an incomplete basal lamina were typically observed. The cells had abundant thin (5 nm) and intermediate (10 nm) myofilaments but thick (15 nm) filaments were never observed.

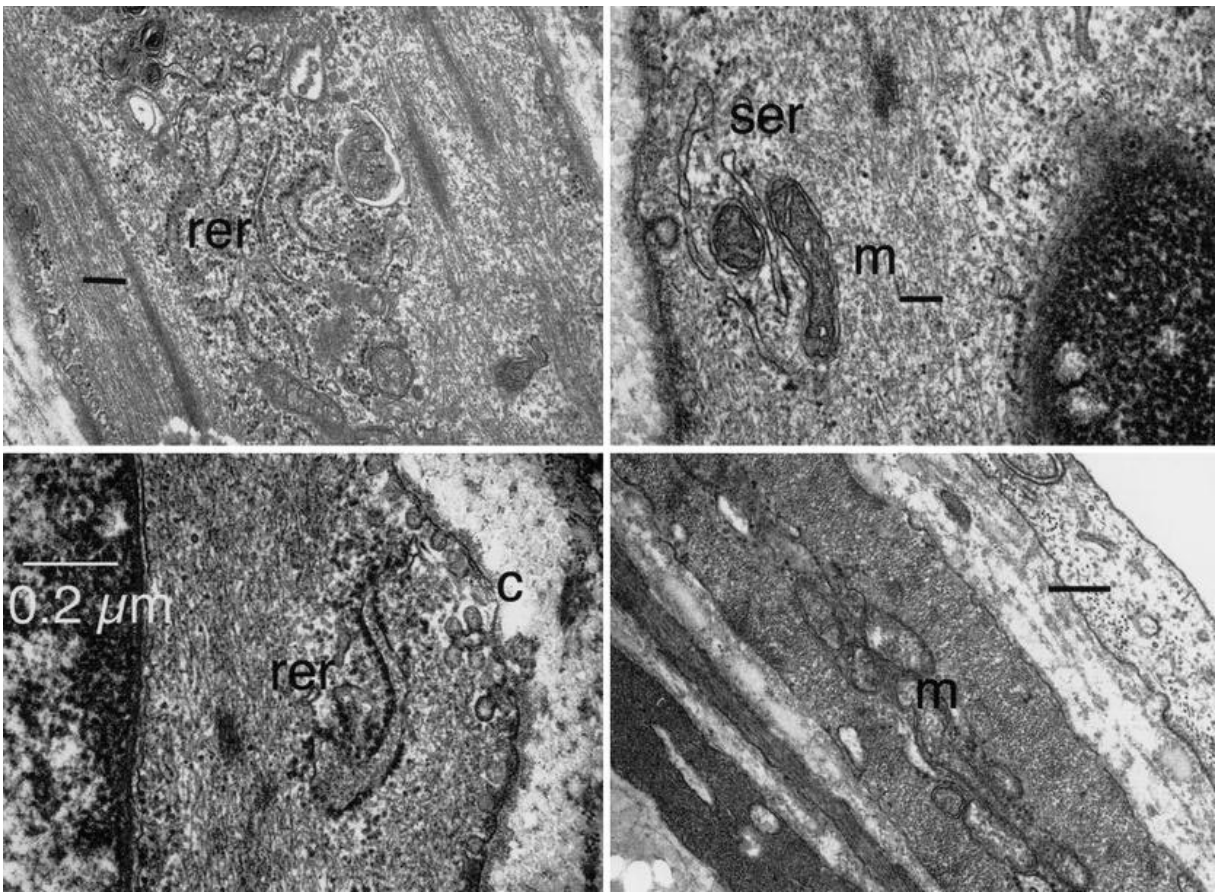

Fig. 2. Higher power sections of the electron-dense cells showing rough endoplasmic reticulum (*rer*), mitochondria (*m*), smooth endoplasmic reticulum (*ser*) and caveolae (*c*). *Scale bar* in each case represents 0.2 μm

Immunohistochemistry

Staining with an antibody to F-actin provided a more comprehensive picture of the arrangement of smooth muscle in the lymphatic wall. Figure 3 shows a confocal image of such a preparation. The smooth muscle cells are grouped together in bundles of five to eight cells, arranged in interweaving bundles orientated in many different directions to give a basket-weave appearance. This unusual arrangement of smooth muscle could easily account for the twisting fashion in which lymphatic vessels contract unlike the peristaltic squeezing action of gastrointestinal smooth muscle which is arranged in distinct circular and longitudinal layers. Lymphatics from four different animals were examined and showed a very similar pattern of smooth muscle orientation to that seen in Fig. 3.

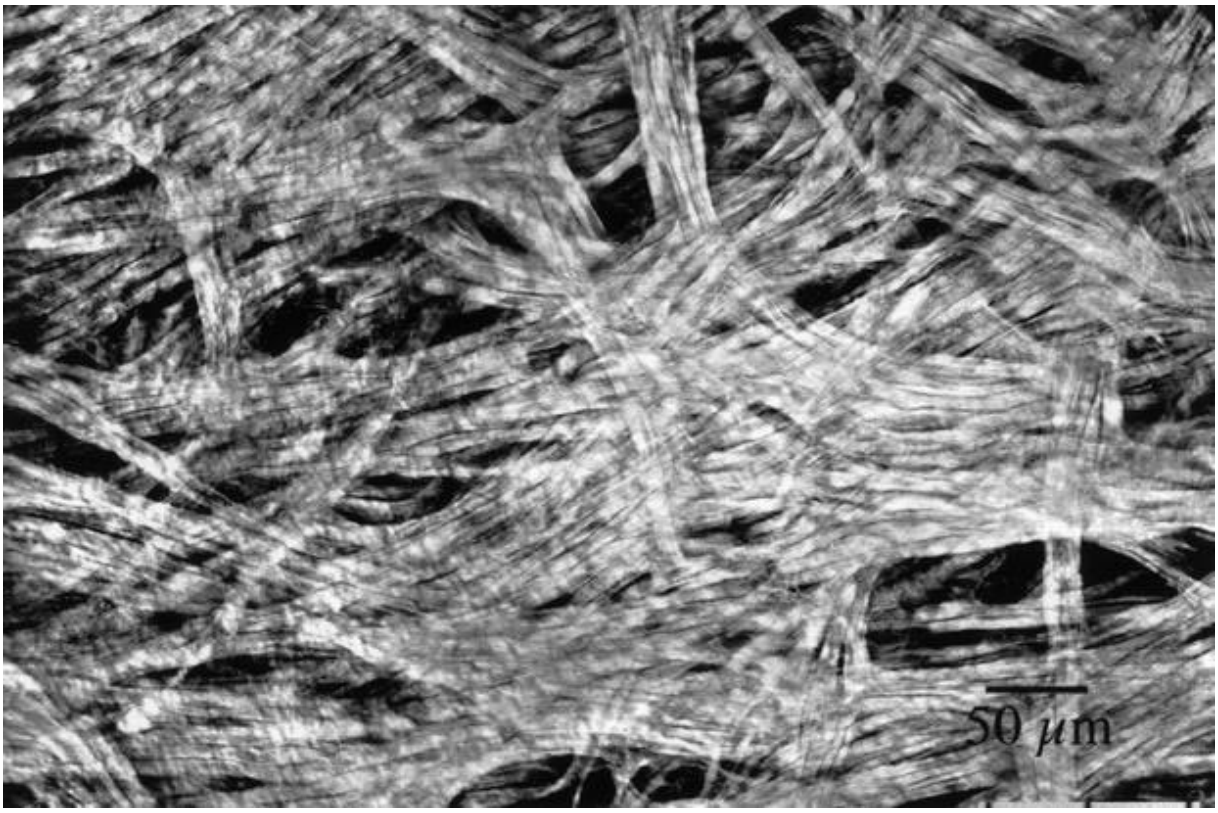

Fig. 3. Confocal image of a whole-mount preparation of lymphatic wall which had been incubated with phalloidin. Smooth muscle cells are arranged in interweaving bundles orientated in many different directions to give a basket-weave appearance. *Scale bar* represents 50 μm

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If the electron-dense cells lying just under the endothelium were indeed similar to the ICC seen in the gastrointestinal tract one would expect to be able to demonstrate this using immunohistochemical techniques. The ICC of the gastrointestinal tract are immunopositive for both Kit and vimentin antibodies and these are widely regarded as a morphological tool for identifying ICC (Ward et al. *1994*, *1995*; Huizinga et al. *1995*; Torihashi et al. *1995*; Burns et al. *1996*). Transverse cryostat sections of lymphatic vessels (from three different animals) were therefore exposed to anti-Kit antibody with the result shown in Fig. 4. Brightly labelled Kit-immunopositive cells were found at the luminal side of the vessel in a position very similar to that occupied by the electron-dense cells in Fig. 1. Similar results were obtained with anti-vimentin antibody but as in the picture shown it was possible only to determine the position of the cells in the wall rather than to resolve their detailed morphology. In order to achieve better resolution of the cells we decided to image whole-mount preparations which had been incubated with either anti-Kit or anti-vimentin antibodies. A Z-series of images was captured beginning from the luminal side of the vessel whereupon long cells became visible in the most superficial layers of the preparation. These could not be confused with endothelial cells since their morphology was completely different. The resultant confocal stacks were reconstructed as three-dimensional shadow projections using Imaris software. Figure 5 *upper panel* shows such a preparation where vimentin-positive cells can be seen lying along the axis of the vessel just beneath the endothelial layer. Cells with a similar morphology and in the same location were also immunopositive for Kit antibody (Fig. 5 *lower panel*). At a higher magnification of this picture one could see that the cells showed a central dilatation in the nuclear region which tapered to a long cylindrical process which could be seen in many cases to be branched. These branches frequently made contact with neighbouring cells to form a network. The cells had a mean length (±SEM) of

174.7±34 µm and a mean diameter at the widest part of 10.7±1.4 µm. In each of the above experiments negative controls were performed by omitting the primary antibody but otherwise adhering to the identical protocol. Tissues or cells treated in this way were in each case invisible in the confocal microscope.

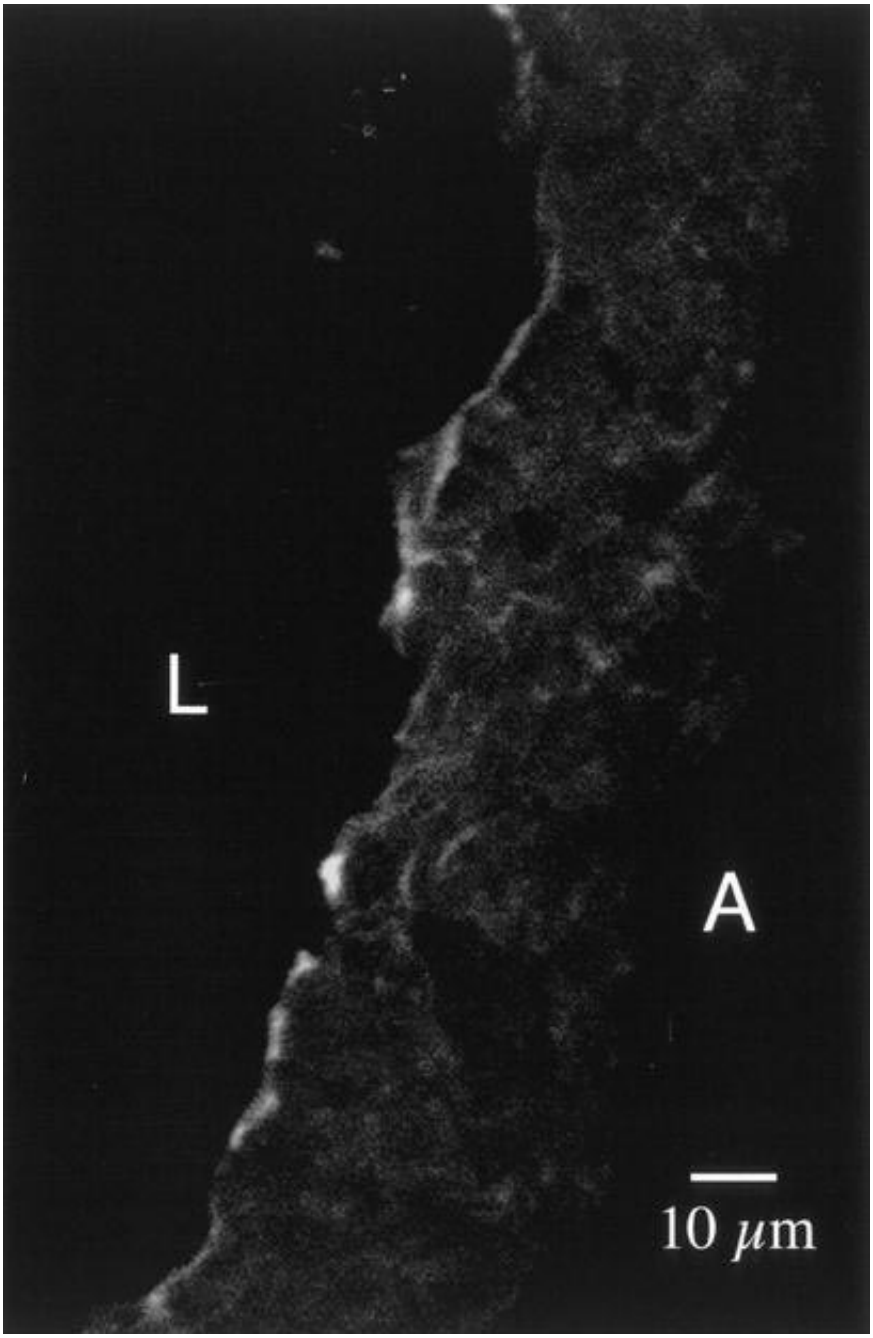

Fig. 4. Transverse cryostat section of the wall of a lymphatic which had been incubated in Kit antibody. Brightly fluorescing cells can be seen at the luminal side (*L*) of the vessel wall. *A* Adventitia. *Scale bar* represents 10 µm

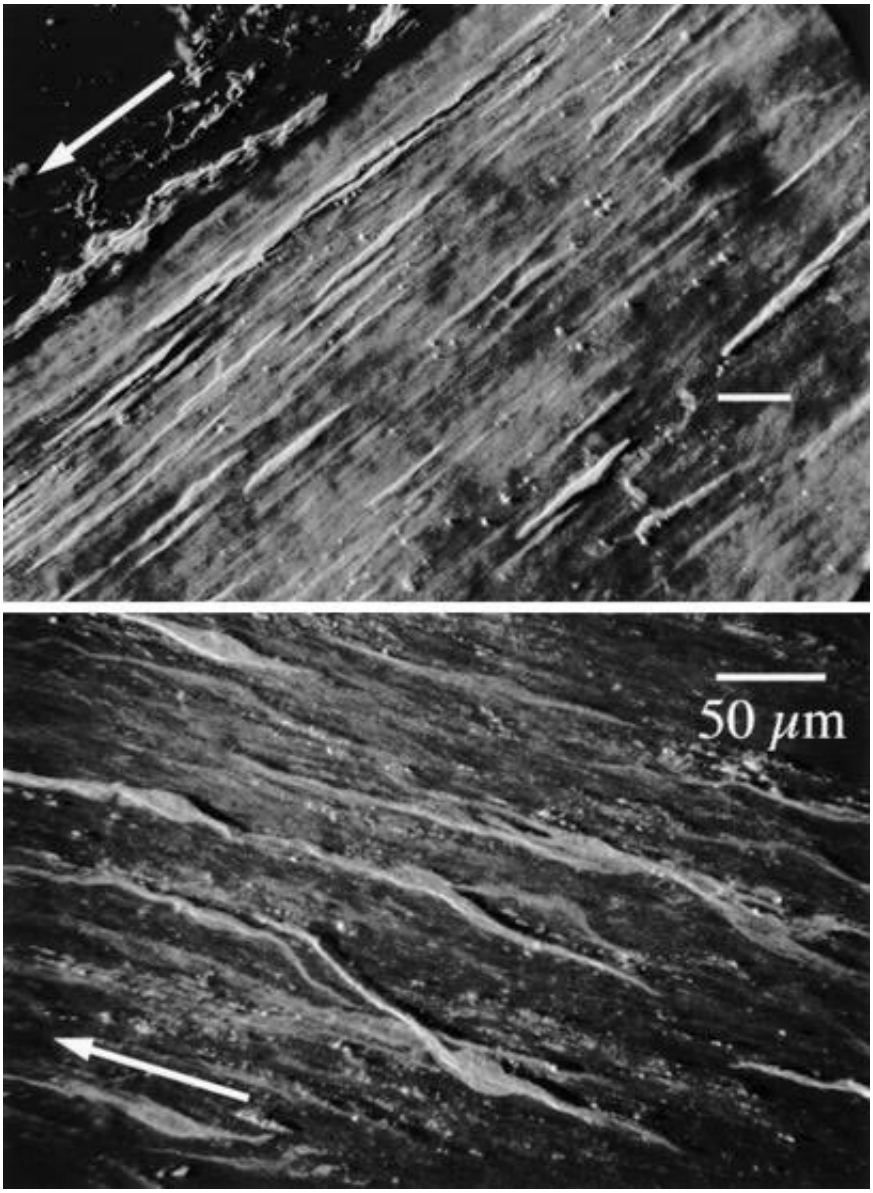

Fig. 5. Shadow projections of confocal stacks of whole-mount preparations incubated in anti-vimentin antibody (*upper panel*) and anti-Kit antibody (*lower panel*) The spindle-shaped cells are seen just below the endothelium and orientated along the vessel axis (indicated by the *arrow*) in both cases. *Scale bar* in each case represents 50 μm

Dispersed cells

The findings of the present study were consistent with the existence of a subset of specialised cells lying just below the endothelium. If the cells which showed pacemaker properties in the electrophysiological studies were the same as those described above one would expect to find them among the products of a routine cell dispersal used for electrophysiological studies. It was therefore of interest to look for them in such a dispersal. Cells normally used for patch-clamp experiments (from a total of eight different animals) were fixed for immunohistochemical investigation and subsequently stained with either vimentin or smooth muscle myosin antibodies. Both types of cell were found in the dispersal. Anti-smooth muscle myosin-positive cells had the typical spindle appearance of smooth

muscle cells (Fig. 6 *right-hand panel*) whereas the vimentin-positive cells could either have several branches, as in the example shown in Fig. 6 *lower left-hand panel*, or could more closely resemble the smooth muscle cells in Fig. 6 *upper left-hand panel*. The majority of cells in any dispersal stained with anti-smooth muscle myosin whereas few stained with anti-vimentin. This was apparent when one compared cells that were visible under brightfield illumination with those seen under fluorescent illumination. However, it was difficult to make a convincing quantitative comparison since a large proportion of cells were lost during processing (typically there were >50 cells at the outset and <10 cells when processing was complete). Attempts were made to double-stain with both anti-myosin and anti-vimentin but the extra processing time involved meant that all the cells had become detached from the coverslip by the end of the procedure.

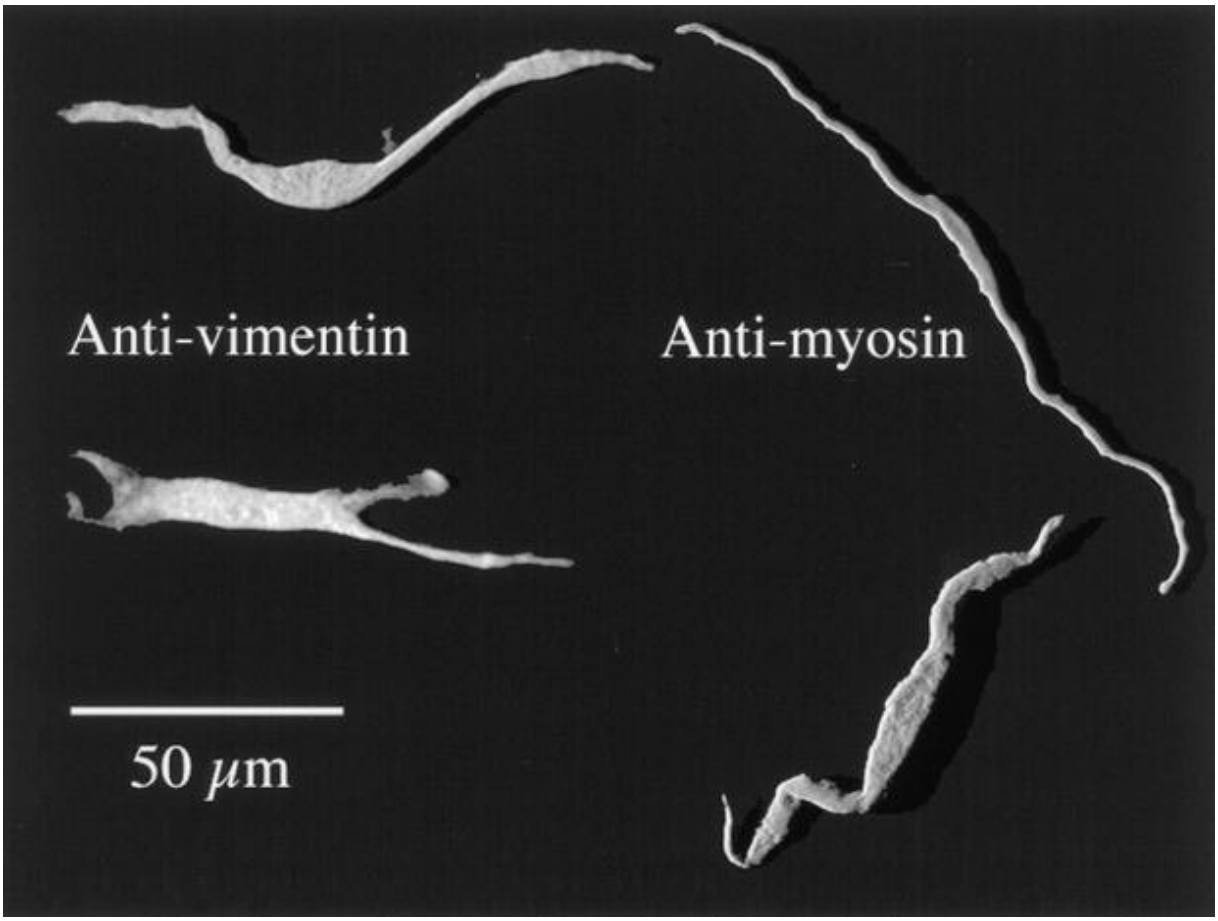

Fig. 6. Shadow projections of isolated cells incubated with anti-vimentin antibody (*left-hand side*) and with anti-smooth muscle myosin (*right-hand side*). Anti-smooth muscle myosin-positive cells had the typical appearance of smooth muscle cells whereas the vimentin-positive cells could either have several branches (*lower left-hand panel*) or could resemble a partially contracted smooth muscle cell (*upper left-hand panel*). *Scale bar* represents 50 μm

Discussion

The present investigation has used electron microscopy and immunohistochemical methods to identify putative pacemaker cells in sheep mesenteric lymphatics. In the gastrointestinal tract, pacemaker cells known as interstitial cells of Cajal have been extensively studied using electron microscopy (Thuneberg *1982*; Rumessen and Thuneberg *1996*), and this was the method used to unequivocally identify these cells prior to 1994. Typically, ICC are electron dense, possess numerous mitochondria, caveolae, rough and smooth endoplasmic reticulum, Golgi apparatus, thin and intermediate filaments, incomplete basal laminae and often also have dense bodies. These ICC have two or more processes and form close associations and gap junctions with each other, smooth muscle cells and nerves. In lymphatic vessels, a ring of electron-dense cells was seen lying just below the endothelium. These cells possessed caveolae, mitochondria, smooth and rough endoplasmic reticulum, dense bodies, a basal lamina and both thin (5 nm) and intermediate (10 nm) filaments. They were distinct from fibroblasts in that they lacked the characteristic dilated cisternae of rough endoplasmic reticulum and possessed caveolae (Sergeant et al. *2000*). In general, the electron-dense cells had many myogenic properties and were reminiscent of the ICC found within the muscle layers of the gastrointestinal tract

(Burns et al. *1996*; Ward et al. *1998*). Interestingly, in previous reports where the ultrastructure of the endothelial area of lymphatic vessels was studied, electron-dense cells similar to those described in the present study can be seen located between the endothelial cells and the smooth muscle layers (Schmid-Schonbein *1990*).

Recently, immunohistochemistry has been employed to reliably identify ICC in tissues of the gastrointestinal tract. Antibodies to the intermediate filament vimentin, found in ICC, as well as a Kit antibody are now widely used as morphological tools to label ICC (Torihashi et al. *1995*; Wang et al. *2000*); *kit* is a proto-oncogene encoding a tyrosine kinase receptor which is now known to be located on ICC (Ward et al. *1994*, *1995*; Huizinga et al. *1995*; Torihashi et al. *1995*). Indeed, treatment of newborn mice with an antibody to *kit* has demonstrated the functional significance of ICC where deficient ICC network formations have been observed leading to gut distension, poor electrical and contractile activity and a loss of peristalsis (Torihashi et al. *1995*; Ward et al. *1997*). Antibodies to *kit* and vimentin were therefore used in the present investigation to identify specialised cells in lymphatic vessel whole mounts. Both of these antibodies labelled cells located just below the lumen which were axially orientated and appeared to contact each other. These cells had prominent oval nuclei and were seen both as branched cells and as cells with a spindle-shaped morphology. They closely resembled the ICC found in the circular and longitudinal muscle layers (IC-IM) of the murine lower oesophageal and pyloric sphincters (Ward et al. *1998*) and the gastric fundus (Burns et al. *1996*).

The smooth muscle content of the wall of the larger lymphatic vessels has been found to be variable between and within different species. Crowe (*1996*) found that guinea-pig mesenteric lymphatics varied in their smooth muscle content between preparations. Morris (*1970*) reported sheep popliteal vessels having between two and five layers of smooth muscle cells while the larger mesenteric vessels imaged in the present investigation had walls averaging ten cells thick. Moreover, the smooth muscle in lymphatic vessels does not appear to be regularly arranged in longitudinal and circular layers as is found in the gut or vascular tissues. Instead, the smooth muscle cells form bundles which interlock to assume a basket-weave formation as was clearly demonstrated in the confocal images of F-actin labelling. This unusual arrangement of smooth muscle has been seen in bovine (Hanley et al. *1992*) as well as guinea-pig mesenteric lymphatics (Crowe *1996*). How the smooth muscle bundles contract to propel lymph along the vessels remains somewhat elusive. If real time recording of smooth muscle cell contraction during spontaneous activity of a lymphatic vessel was achievable, this would enable us to observe how these interwoven bundles work together to generate coordinated contractions. Ultrastructurally, lymphatic smooth muscle cells had properties typical of all smooth muscle preparations. Dense bodies, abundant thick and thin filaments, caveolae, mitochondria, smooth endoplasmic reticulum and a complete basal lamina were common to all the cells. In addition, they were well coupled to each other by frequent gap junctions and close associations. This was unsurprising as previous functional studies concluded that the smooth muscle cells must be electrically coupled in order to produce such coordinated phasic activity (Van Helden *1993*) and the use of pharmacological blockers of gap junctions has been shown to disrupt coordinated contractions in pumping lymphatic vessels (McHale and Meharg *1992*; Zawieja et al. *1993*).

We are still at an early stage in our understanding of the nature of pacemaking in lymphatic vessels. What we do know is that the pacemaker currents found in the sinu-atrial node of the heart [I_f and $I_{Ca(T)}$] are found in only a very small proportion of cells isolated by collagenase dispersal from sheep mesenteric lymphatics. In the case of I_f these accounted for less than 5% of any given dispersal which made it necessary to patch-clamp a very large number of cells (more than 800) in order to study the current adequately (McCloskey et al. *1999*). In the case of $I_{Ca(T)}$ the proportion of cells showing the current was also less than 5% (unpublished observations) but, partly due to the time commitment involved, a similarly detailed study of this current has not been done. It had been our hope, when we

discovered that a small proportion of the cells in the lymphatic wall were morphologically and immunologically distinct from the bulk of smooth muscle cells, that it would be possible, with suitable in vivo labelling, to patch-clamp only these cells. This has not as yet been possible since any effective label has also rendered the cells non-viable for voltage clamp. Nevertheless, we have demonstrated for the first time in the vascular bed that lymphatic vessels contain a subpopulation of cells lying below the endothelium which have ultrastructural characteristics similar to those found in pacemaker cells of other tissues. They are vimentin and Kit positive and occur in about the same proportions as the lymphatic cells that showed pacemaking properties in electrophysiological studies and they are of similar appearance and dimensions.

Acknowledgements. The authors would like to thank the Anatomy Department QUB for the use of the confocal microscope and J.W. Robinson and Sons, Lurgan, for supplying the tissue. This work was supported by grants from the British Heart Foundation and the European Union.

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