*British Journal of Surgery* 1994, **81**, 819–822

# Influence of Losartan, an angiotensin receptor antagonist, on neointimal proliferation in cultured human saphenous vein

K. VARTY, K. E. ALLEN, L. JONES*, R. D. SAYERS, P. R. F. BELL and N. J. M. LONDON

*Departments of Surgery and *Pathology, Clinical Sciences Building, University of Leicester, Leicester LE2 7LX, UK*
*Correspondence to: Mr K. Varty*

**An organ culture of human saphenous vein was used as a model of vein graft intimal hyperplasia and the potential of Losartan, an angiotensin II receptor antagonist, to inhibit neointimal proliferation was investigated. Median (range) neointimal thickness was reduced from 17 (16–19) to 11 (8–18) μm in veins cultured with Losartan (median difference 5 (95 per cent confidence interval 2–8) μm). A similar decrease in the median neointimal proliferation index was seen from 21 (range 14–47) to 15 (range 5–31) per cent (median difference 8 per cent (95 per cent confidence interval 5–11 per cent)). These results demonstrate that angiotensin II receptor antagonists may be of therapeutic value for the modulation of vein graft intimal hyperplasia.**

Advanced intimal hyperplasia encroaching on the vessel lumen leads to stenosis in 20 per cent of lower-limb vein grafts[1]. These lesions pose a threat to subsequent patency, demanding intensive graft surveillance for early detection. The final common pathway in the formation of such stenosis is smooth muscle cell proliferation, which is regulated by a complex interaction of mitogens. Since Dzau[2] demonstrated the presence of a local renin–angiotensin system within the vascular wall, there has been considerable interest in the role of angiotensin II in the regulation of intimal hyperplasia. Powell and colleagues[3] were first to describe how angiotensin-converting enzyme (ACE) inhibitors could significantly reduce intimal hyperplasia in injured arteries. Others reported that proliferating smooth muscle cells in the neointima express angiotensin II receptors[4] and messenger RNA for angiotensinogen[5].

The role of the local renin–angiotensin system in the development of vein graft intimal hyperplasia has been less intensively studied and remains unclear. The results of two recent *in vivo* animal studies, however, suggest that angiotensin II may also act as a significant smooth muscle cell mitogen in such cases. O'Donohoe and colleagues[6] reported a reduction in intimal thickening (from 150 to 90 μm) in rabbit jugular vein grafts following a 28-day course of the ACE inhibitor captopril. A smaller, but still significant, decrease (from 132 to 105 μm) was also observed in canine vein grafts after a 42-day treatment with cilazapril[7].

In the present study an organ culture of human saphenous vein was used to model vein graft intimal hyperplasia, as first described by Soyombo and colleagues[8]. The influence of Losartan (Du Pont, Wilmington, Delaware, USA), a non-peptide angiotensin II receptor antagonist, on the neointimal proliferation occurring in this model was investigated.

## Materials and methods

*Vein culture preparation*

Segments of long saphenous vein were obtained from patients undergoing coronary artery or lower-limb artery bypass grafting, or carotid endarterectomy in which the vein was used as the bypass conduit or as a patch. Patients who had recently taken or were currently being prescribed ACE inhibitors were excluded. Local ethics committee approval was obtained. The length of vein available was dictated by clinical requirements and was usually 2–3 cm. This was exposed and dissected using a 'no-touch' technique and the tributaries tied with 3/0 silk. Distension was avoided in all cases and handling kept to a minimum. The vein was placed immediately into cold (4°C) calcium-free Krebs solution (composition: NaCl 118 mmol/l, KCl 4·7 mmol/l, $MgSO_4$ 1·2 mmol/l, $KH_2PO_4$ 1·2 mmol/l, $NaHCO_3$ 25 mmol/l, glucose 11·1 mmol/l) and transported to the laboratory.

The vein culture system was based on that developed by Soyombo and colleagues[8], a modification of the earlier methods of Pederson and Bowyer[9]. The excess adventitial tissue was removed with great care from the vein segments under aseptic conditions while still immersed in cold calcium-free Krebs solution using fine dissecting scissors and forceps. The potentially damaged ends of the vein were excised with a sharp size 23 scalpel blade and discarded. The remaining vein was divided into 5-mm segments, which were then cut open longitudinally. One segment was routinely stained with 0·2 per cent aqueous trypan blue for 30 s at room temperature to allow rapid assessment of endothelial coverage. Veins with over 50 per cent trypan blue staining, indicating excessive endothelial injury and loss, were excluded from the study. The sections were transferred into vein culture dishes. These were made up using 60 × 20 mm Pyrex dishes (Dow Corning, Reading, UK) into which Sylgard 184 resin (Dow Corning, Seneffe, Belgium) was added to a depth of approximately 5 mm. The vein sections were stretched out, luminal surface uppermost, and pinned on to a coarse (500-μm) polyester cloth in the dishes using fine minuten pins. The vein was then immersed in 5 ml vein culture medium and placed in a humidified cell culture incubator at 37°C with an atmosphere of 5 per cent carbon dioxide in air.

The vein culture medium comprised: RPMI 1640 tissue culture medium (Northumbria Biologicals, Cramlington, UK); 30 per cent fetal calf serum (Imperial Laboratories, Andover, UK); penicillin 50 units/ml; streptomycin 50 μg/ml; endothelial cell growth factor (Sigma Chemicals, Poole, UK) 15 μg/ml; and L-glutamine 2 mmol/l. The culture medium was changed every 48–72 h.

*Study design*

Three 5-mm sections were taken from each of ten veins. One section served as the preculture baseline. This was pinned out in a vein culture dish, covered with 10 per cent formal saline for 12 h and then processed for histological analysis. The second section served as a control. This was cultured in standard medium to which 100 μl sterile distilled water (drug vehicle) were added. The final section of vein was cultured with Losartan at a concentration of $10^{-5}$ mol/l in 100 μl vehicle. The activity of the Losartan was checked by its ability to abolish the contractile response of uncultured saphenous vein to angiotensin II in an organ bath

Presented to the Association of Surgeons of Great Britain and Ireland in Birmingham, UK, April 1993
Paper accepted 5 October 1993

preparation. Medium, drug and vehicle were changed every 48–72 h. All veins were cultured for 14 days.

The specificity of the action of Losartan was evaluated by culturing four veins with both Losartan and angiotensin II. The above protocol was followed with the exception that angiotensin II ($10^{-5}$ mol/l) was also added to the medium of the vein cultured with Losartan. Angiotensin II was dissolved in sterile distilled water and frozen at −20°C in 100-$\mu$l aliquots. This volume was added twice daily to the medium.

*Outcome measures*

Proliferating cells of all cultured veins were labelled with 5-bromo-2′-deoxyuridine (BrdU; Amersham, High Wycombe, UK) for the final 96 h. At the end of this period all veins were covered with 10 per cent formal saline for 12 h and processed for histological examination. A combined Miller's elastin and monoclonal smooth muscle actin stain was applied to identify the layers of the vein wall. This is a novel combination involving an indirect immunoperoxidase method for localization of smooth muscle actin. Mouse antihuman $\alpha$ smooth muscle actin antibody (Dako, High Wycombe, UK) was used at 1:400 with diaminobenzidine as a final reaction product. After this a Miller's elastin stain[10] was performed. This stain revealed a neointima of smooth muscle actin-positive cells in cultured veins (*Fig. 1*). The thickness of this layer was measured by two independent observers using a computerized image analysis system (Kontron Videoplan, Munich, Germany). Thirty measurements were made on each vein evenly distributed across the whole section.

Labelled proliferating cells were localized in 4-$\mu$m histological sections. BrdU was detected with a monoclonal antibody (Europath, Bude, UK) using the avidin-binding complex technique. Counts of the number of labelled neointimal cells in each high-power field (×320) were made across the vein section by two independent observers, and the mean proportion used as the final proliferation index.

*Statistical analysis*

All summary data are expressed as median (range). Differences between paired veins were compared with the Wilcoxon paired rank sum test.

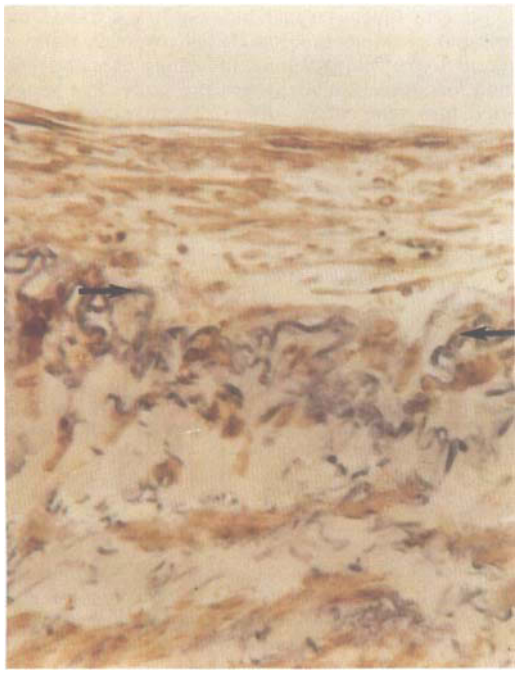

**Fig. 1** Histological section of cultured vein stained for smooth muscle actin and elastin. A cellular neointima is seen, staining positively for actin, separated from the media by the internal elastic lamina (arrows). (Original magnification × 160)

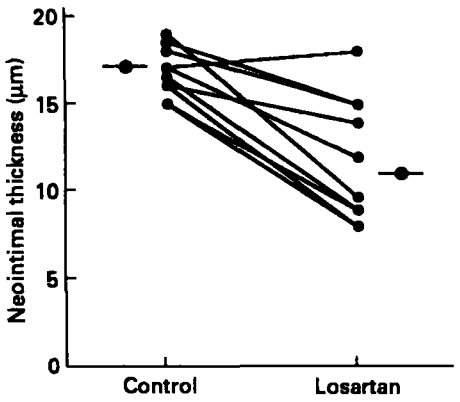


**Fig. 2** Neointimal thickness in ten paired cultured vein segments; $10^{-5}$ mol/l Losartan *versus* control. Bars are medians

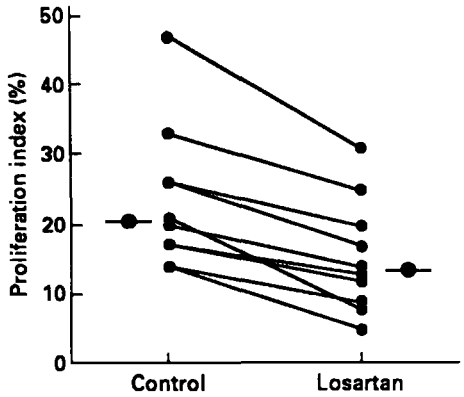


**Fig. 3** Neointimal proliferation index in ten paired cultured vein segments; $10^{-5}$ mol/l Losartan *versus* control. Bars are medians

## Results

The neointimal thickness and proliferation index for control veins and those cultured with Losartan are shown in *Figs 2* and *3*. A significant reduction in median neointimal thickness from 17 (range 16–19) to 11 (range 8–18) $\mu$m was observed in veins cultured with Losartan (median difference 5 (95 per cent confidence interval 2–8) $\mu$m; $P = 0{\cdot}008$). A similar decrease in the median neointimal proliferation index was seen from 21 (range 14–47) to 15 (range 5–31) per cent in veins cultured with Losartan (median difference 8 per cent (95 per cent confidence interval 5–11 per cent); $P = 0{\cdot}005$).

In veins cultured with $10^{-5}$ mol/l Losartan plus $10^{-5}$ mol/l angiotensin II there was no significant difference in median neointimal thickness (20 (range 16–22) *versus* 18 (range 15–22) $\mu$m) nor median proliferation index (21 (range 18–23) *versus* 22 (range 20–23) per cent) compared with control veins.

## Discussion

The aim of this study was to assess the role of the renin–angiotensin system in the neointimal proliferative response in cultured human saphenous vein. The specific angiotensin II receptor antagonist Losartan was used as it has been proposed that local vascular angiotensin II production may involve a non-ACE dependent pathway[11]. Losartan is a non-peptide agent that overcomes several of the problems of previous peptide antagonists (such as saralasin), which could

not be given orally, had a short duration of action and some partial agonistic activity. Trials in human volunteers have demonstrated the safety and efficacy of Losartan in reversing angiotensin II-induced pressor responses[12]. Wong *et al.*[13] have reported that the potent metabolite EXP3174 is responsible for a significant component of the *in vivo* activity of Losartan. Accurate reproduction of the *in vivo* effect of Losartan *in vitro* is, therefore, difficult. In the present study the concentration of Losartan was the same as that described by Chiu and colleagues[14] in smooth muscle cell culture experiments, which approximates with peak serum levels observed in normal human volunteers.

Losartan produced a reduction of approximately 33 per cent in both neointimal thickness and proliferation index, an effect that could be reversed with angiotensin II. This confirms that Losartan is acting via blockade of angiotensin II receptors and not through some other non-specific effect. The source of angiotensin II in the vein culture model is unknown, but cultured endothelial cells produce this substance and seem therefore to be the most likely source[15]. Neointimal proliferation occurring in cultured saphenous vein is promoted by the endothelium[16] and angiotensin II therefore represents one of the potential messengers involved in this process.

As a model of vein graft intimal hyperplasia, vein culture produces a neointimal layer closely resembling that seen *in vivo*. The stimuli for the production of this layer *in vitro*, however, are likely to differ from those present *in vivo*. In particular, the vein culture system used for the present study does not incorporate flow, which has an important influence on hyperplasia *in vivo*, low flow leading to an increase[17]. The model possibly represents the extreme situation where the absence of flow acts as a stimulus for intimal proliferation. The endothelium plays a major role in detecting and translating changes in flow into vessel wall responses *in vivo*[18] and is also responsible for the neointimal proliferation that occurs *in vitro* in this model[16].

The magnitude of the response seen in the present study is comparable to that seen in *in vivo* animal studies with Losartan and ACE inhibitors. Two studies of Losartan in rats after arterial injury reported reduced intimal thickening of 38 per cent[19] and 49 per cent[20]. In animal vein grafts, ACE inhibitors produced a decreased intimal thickness of 40 per cent[6] and 21 per cent[7]. These responses to blockade of angiotensin II activity on the neointimal smooth muscle cell are also comparable to those seen when monoclonal antibodies are used to inhibit the action of polypeptide growth factors. Administration of an antibody to platelet-derived growth factor (PDGF) in rats following arterial injury reduced the intimal area by 41 per cent[21]. An antibody to basic fibroblast growth factor (bFGF) in the same model led to a marked decrease in early smooth muscle cell proliferation rate and a reduction in intimal thickness of 37 per cent from 75 to 47 $\mu$m at 8 days after injury[22]. These observations suggest that each smooth muscle cell mitogen (angiotensin II, PDGF, bFGF) plays a part in the intimal hyperplastic response, but that none is dominant. Rather, an additive or synergistic interaction between mitogens has been suggested[23]. One consequence of this hypothesis is that more than one therapeutic agent may be necessary to achieve adequate control of intimal hyperplasia. The present study demonstrates that angiotensin II receptor antagonists may have a significant role to play as components of such combination therapy aimed at combatting vein graft intimal hyperplasia. The synergistic action between ACE inhibitors and heparin has been well documented[24,25]; studies are required to see whether a similar synergism exists between Losartan and heparin.

## Acknowledgements

This work was supported by grants from the British Heart Foundation and the Mason Medical Research Foundation.

## References

1 Varty K, Allen KE, Bell PRF, London NJM. Infrainguinal vein graft stenosis. *Br J Surg* 1993; **80**: 825–33.
2 Dzau VJ. Circulating *versus* local renin–angiotensin system in cardiovascular homeostasis. *Circulation* 1988; 77(Suppl I): I4–13.
3 Powell JS, Clozel JP, Rita KM *et al.* Inhibitors of angiotensin converting enzyme prevent myointimal proliferation after vascular injury. *Science* 1989; **245**: 186–8.
4 Viswanathan M, Stromberg C, Seltzer A, Saavedra JM. Balloon angioplasty enhances the expression of angiotensin II $AT_1$ receptors in neointima of rat aorta. *J Clin Invest* 1992; **90**: 1707–12.
5 Rakugi H, Jacob HJ, Ingelfinger JR, Krieger JE, Dzau VJ, Pratt RE. Angiotensinogen gene expression in the myointima after vascular injury. *Hypertension* 1990; **16**: 345.
6 O'Donohoe MK, Schwartz LB, Radic ZS, Mikat EM, McCann RL, Hagen PO. Chronic ACE inhibition reduces intimal hyperplasia in experimental vein grafts. *Ann Surg* 1991; **214**: 727–32.
7 Hirko MK, McShannic JR, Sharp WV. Pharmacologic modulation of intimal hyperplasia in canine interposition grafts. *J Vasc Surg* 1992; **16**: 472–3.
8 Soyombo AA, Angelini GD, Bryan AJ, Jasani B, Newby AC. Intimal proliferation in an organ culture of human saphenous vein. *Am J Pathol* 1990; **137**: 1401–10.
9 Pederson DC, Bowyer DE. Endothelial injury and healing *in vitro*. Studies using an organ culture system. *Am J Pathol* 1985; **119**: 264–72.
10 Miller RJ. An elastin stain. *Med Lab Tech* 1971; **28**: 148–54.
11 Okunishi H, Miyazaki T, Okamura T, Toda N. Different distribution of two types of angiotensin II generating enzymes in the aortic wall. *Biochem Biophys Res Commun* 1987; **149**: 1186–92.
12 Christen Y, Waeber B, Nussberger J, Lee RJ, Timmermans PBMWM, Brunner HR. Dose–response relationships following oral administration of DuP 753 to normal humans. *Am J Hypertens* 1991; **4**: 350–4S.
13 Wong PC, Price WA, Chiu AT *et al.* Nonpeptide angiotensin II receptor antagonists. XI. Pharmacology of EXP3174: an active metabolite of DuP 753, an orally active antihypertensive agent. *J Pharmacol Exp Ther* 1991; **255**: 211–17.
14 Chiu AT, Roscoe WA, McCall DE, Timmermans PBMWM. Angiotensin II-1 receptors mediate both vascoconstrictor and hypertrophic responses in rat aortic smooth muscle cells. *Receptor* 1991; **1**: 133–40.
15 Kifor I, Dzau VJ. Endothelial renin–angiotensin pathway: evidence for intracellular synthesis and secretion of angiotensins. *Circ Res* 1987; **60**: 422–8.
16 Angelini GD, Soyombo AA, Newby AC. Smooth muscle proliferation in response to injury in an organ culture of human saphenous vein. *Eur J Vasc Surg* 1991; **5**: 5–12.
17 Dobrin PB, Littooy FN, Endean ED. Mechanical factors predisposing to intimal hyperplasia and medial thickening in autogenous vein grafts. *Surgery* 1989; **105**: 393–400.
18 Kohler TR, Kirkman TR, Kraiss LW, Zierler BK, Clowes AW. Increased blood flow inhibits neointimal hyperplasia in endothelialized vascular grafts. *Circ Res* 1991; **69**: 1557–65.
19 Farhy RD, Ho K, Carratero OA, Scicli AG. Kinins mediate the antiproliferative effect of ramipril in rat carotid artery. *Biochem Biophys Res Commun* 1992; **182**: 283–8.
20 Prescott MF, Webb RL, Reidy MA. Angiotensin-converting enzyme inhibitor *versus* angiotensin II $AT_1$ receptor antagonist. Effects on smooth muscle cell migration and proliferation after balloon catheter injury. *Am J Pathol* 1991; **139**: 1291–6.
21 Ferns GAA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 1991; **253**: 1129–32.

22 Lindner V, Reidy MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci U S A* 1991; **88**: 3739–43.
23 Sharefkin JB. Drug effects on vascular wall cell gene expression: a probe of the molecular biology of the early hyperplasia/late fibrosis response to large vessel injury. *J Vasc Surg* 1992; **15**: 921–3.
24 Powell JS, Muller KM, Baumgartner HR. Suppression of the vascular response to injury: the role of angiotensin converting enzyme inhibitors. *J Am Coll Cardiol* 1991; **17**: 137B–42B.
25 Clowes AW, Clowes MM, Vergel SC *et al.* Heparin and cilazapril together inhibit injury induced intimal hyperplasia. *Hypertension* 1991; **18**(Suppl II): II65–9.

*British Journal of Surgery* 1994, **81**, 822–823

## Short note

# Colour Doppler ultrasonographic imaging in the diagnosis of popliteal artery entrapment syndrome

S. T. R. MacSWEENEY, R. CUMING and R. M. GREENHALGH

*Department of Surgery, Charing Cross and Westminster Hospital Medical School, Fulham Palace Road, London W6 8RF, UK*
*Correspondence to: Mr S. T. R. MacSweeney*

Popliteal artery entrapment syndrome occurs when an abnormal anatomical relationship between the popliteal artery and the surrounding musculotendinous structures causes repeated arterial compression on exercise[1,2]. Claudication and eventually irreversible damage to the popliteal artery, with thrombosis, embolization and aneurysmal dilatation, may result, producing ischaemia and threatening limb viability. Diagnostic delay is common and increases morbidity. Colour Doppler ultrasonographic imaging may be useful in the early diagnosis of this condition.

## Patients and methods

The patient lies prone and the popliteal artery is imaged with a 5-MHz probe on an Acuson 128 instrument (Acuson, Mountain View, California, USA). Structural damage to the artery can be visualized (*Fig. 1*), but before such damage is present the artery may appear normal at rest and, unless manoeuvres that reproduce arterial compression are performed, the condition may not be diagnosed (*Fig. 2*). Such manoeuvres include active plantar flexion against resistance and passive dorsiflexion of the foot. As the calf muscles contract the popliteal artery is compressed and blood flow reduced.

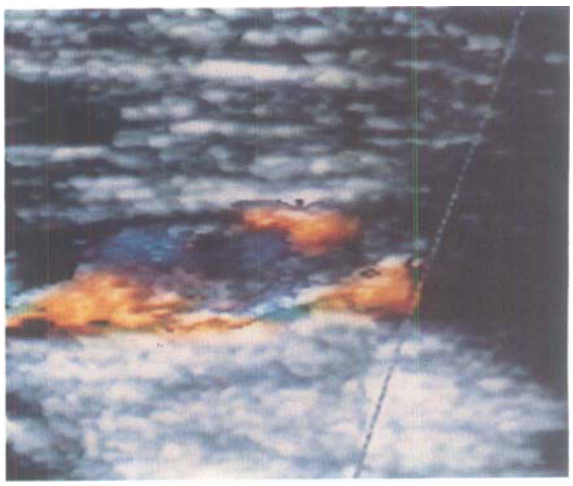

**Fig. 1** Colour Doppler ultrasonographic image showing the lumen of an entrapped popliteal artery partially filled with loose thrombus. Blood (red) can be seen flowing around the thrombus, which tends to fragment causing distal embolization

Paper accepted 10 October 1993

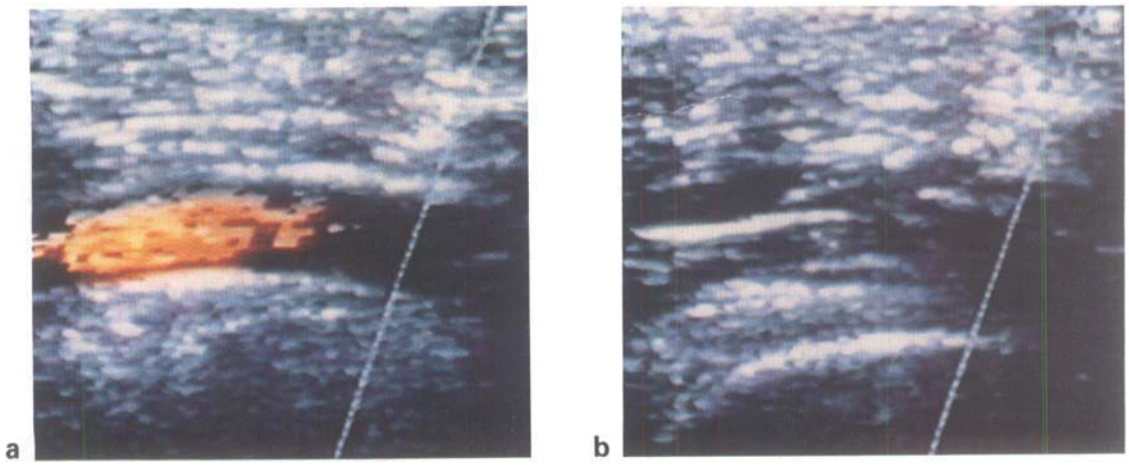


**Fig. 2** **a** Colour Doppler ultrasonographic image of an apparently normal popliteal artery at rest with blood flowing through it. **b** When entrapment is unmasked by resisted plantar flexion the lumen is occluded by extrinsic compression and blood flow ceases