Our aim was to develop a clinically relevant model of atrophic nonunion in the rat to test the hypothesis that the vessel density of atrophic nonunion reaches that of normal healing bone, but at a later time-point. Atrophic nonunion is usually attributed to impaired blood supply and is poorly understood.

We determined the number of blood vessels at the site of an osteotomy using immunolocalisation techniques in both normally healing bones and in atrophic nonunion. At one week after operation there were significantly fewer blood vessels in the nonunion group than in the healing group. By eight weeks, the number in the atrophic nonunion group had reached the same level as that in the healing group.

Our findings suggest that the number of blood vessels in atrophic nonunion reaches the same level as that in healing bone, but at a later time-point. Diminished vascularity within the first three weeks, but not at a later time-point, may prevent fractures from uniting.

Although atrophic nonunion is usually attributed to impaired blood supply, both experimental and clinical studies have shown that vascularity is not reduced in established atrophic nonunion.1-7 The number of blood vessels, however, has not been determined at the early stages.

Animal models of healing fractures and nonunion are of value for assessing parameters at time-controlled intervals. Many models of atrophic nonunion rely on large segmental excisions.8-10 Others use silastic spacers to plug or wrap around the bone-ends.11,12 None of these reflects the clinical situation.

A recent, clinically relevant model of atrophic nonunion has been developed in the rabbit, in which the periosteum and endosteum are stripped for a distance equivalent to one diameter of the tibia both proximally and distally of an osteotomy gap of 2 mm.13 Since rabbits are expensive and often difficult to handle, the development of a small animal model would be of value. Rats are inexpensive, easy to procure, maintain and to handle, they are not as small as mice and application of an external fixator is easy.
Our aim was to develop such a model of atrophic nonunion which did not require a large segmental excision or the introduction of foreign material and which more closely resembled the clinical situation. In addition, we wished to assess the number of blood vessels at the site of nonunion in order to test the hypothesis that the vessel density in atrophic nonunion reaches that of normal healing bones, but at a later time-point.

Materials and Methods

We used 28 adult female Wistar rats randomised into two groups of 14 (nonunion and healing). They were individually caged and allowed water and food ad libitum and unrestricted weight-bearing. After surgery, they were killed at 1, 3, 8 or 16 weeks (Table I) and the right (operated) tibia was prepared for histological examination.

Operative technique. We developed a novel circular frame external fixator using nylon rings and copper screws (Fig. 1). We applied the fixator to the right tibia under general anaesthesia and with aseptic conditions. Eight 27-gauge needles with the flange removed were drilled percutaneously into the proximal and distal metaphyses of the tibia and fixed to the rings of the fixator with an epoxy resin glue. An osteotomy was then performed using a 1 mm burr under constant irrigation with cold saline solution, centrally between the two inside rings of the fixator. The fibula was fractured manually using a three-point bending method. In 14 of the 28 animals we stripped the periosteum and curetted the intramedullary canal for a distance of one tibial diameter, both proximally and distally. A 1 mm gap was introduced at the site of the osteotomy in both the control and experimental groups. The wound was washed thoroughly and the skin closed.

Radiological examination. At any one time two independent senior orthopaedic surgeons (AS, SI, HB) assessed standardised radiographs obtained after operation and every two weeks thereafter. They categorised the fractures as healing or not according to the criteria of the AO-ASIF manual.14 Formation of callus was assessed by scanning all the radiographs into a Macintosh Quadra 650 computer and analysing images (Optilab pro v2.5, Graftek, France). The outline of the callus was traced manually and the size calculated. The results were expressed as a percentage change in

Fig. 2a
Radiograph at 16 weeks after operation showing atrophic nonunion in the stripped group with no formation of callus and rounded bone-ends.

Fig. 2b
Radiograph at 16 weeks after operation in the control (non-stripped) group showing that the tibia has united.

Fig. 3
The median increase or decrease of mineralised tissue (%) between healing fractures and those with nonunion.
the amount of mineralised tissue from the postoperative radiographs.

**Histological examination.** With the external fixator still attached, we fixed the right lower limbs in neutral phosphate-buffered formalin (4% v/v) for 48 hours and then decalcified them in neutral EDTA for six weeks with twice-weekly changes of EDTA. Once decalcification had been confirmed radiologically, we removed the external fixator and processed the samples. They were then embedded in paraffin wax and 6 \( \mu \text{m} \) sections were cut and stained with haematoxylin and eosin.

We assessed the general morphology and the different tissue types present using light microscopy. Capture using a camera and image-grabber software, produced images of the interfragmentary gap at a magnification of x 100. A 10 x 10 grid was placed over the image on the screen. This
applied 100 cross points to a field of view corresponding to an area of 0.31 mm². By systematic examination and counting of the number of crosspoints which overlaid each different tissue type, we were able to calculate the relative proportions of bone, cartilage, fibrous tissue and haematoma.

**Immunohistochemistry.** We used a monoclonal antibody against smooth muscle actin (InnoGenex, San Ramon, California) to quantify the number of blood vessels.

Paraffin sections (6 µm thick) were mounted onto poly-L-lysine-coated slides, dewaxed and rehydrated. A blocking agent of 10% goat serum was added for 15 minutes at room temperature. The anti-smooth muscle actin antibody was diluted in triethanolamine-buffered saline (TBS) to make a working concentration of 5 µg/ml and applied for 60 minutes at room temperature and then washed thoroughly in TBS. Goat anti-mouse alkaline phosphatase-conjugated secondary antibody (Dako, Ely, UK) 1:50 (v/v) was applied for 30 minutes and washed thoroughly in TBS again. For visualisation, fast-red substrate was applied for 30 minutes and washed thoroughly in TBS. Goat anti-mouse alkaline phosphatase-conjugated secondary antibody (Dako, Ely, UK) 1:50 (v/v) was applied for 30 minutes and washed thoroughly in TBS again. After rinsing, the slides were mounted using Aquamount (BDH Merck, Poole, UK). Using light microscopy we counted the total number of blood vessels within the interfragmentary gap and the number within the intracortical region (interfragmentary tissue between the bone cortices) and also in the central regions (interfragmentary tissue not between the bone cortices).

**Statistical analysis.** We determined the differences in the area of callus and vessel density between the groups using confidence intervals (CI) and used a Statview software package (SAS Institute Inc, Cary, North Carolina) for all statistical analyses.

**Results**

**Radiological.** All the animals which had periosteal stripping and curettage of the intramedullary canal went on to form atrophic nonunion at eight and 16 weeks after osteotomy (Fig. 2a) while the other 14 had successful union (Fig. 2b). There was no interobserver variability. There was significantly less formation of callus in the nonunion than in the healing group from four weeks onwards (Fig. 3). In the healing group, the size of the callus initially increased until, at approximately ten weeks, it decreased as remodelling occurred. In the nonunion group the mineralised tissue decreased from four weeks onwards in the eight-week group and from two weeks onwards in the 16-week group.

**Histological examination.** One week after operation, in the stripped group, haematoma filled the interfragmentary gap. In the non-stripped group, the interfragmentary tissue appeared to be more cellular, containing both granulation tissue and fibrous tissue, the bone ends were viable because of the presence of osteocytes and there was a periosteal reaction.

At three weeks after surgery, fibrous tissue filled the interfragmentary gap in both groups. In the stripped group, there were osteoclasts along the surfaces of areas of non-viable bone (Figs 4a and 4b), muscle fibres infiltrating the interfragmentary gap and rounding of the bone-ends. In the non-stripped group, the periosteal callus had increased in size and there were large areas of cartilage in which endochondral ossification was occurring (Figs 4c and 4d).

At eight and 16 weeks after operation, there was a clear difference between the groups. The stripped group had a clear interfragmentary gap where the bone-ends were rounded and the intramedullary canal had become capped off (Figs 5a and 5b), whereas the non-stripped group had full bridging callus (Figs 5c and 5d). There was, however, a difference in the interfragmentary tissue in the nonunion group between eight and 16 weeks. At eight weeks, fibrous tissue filled the interfragmentary gap, but at 16 weeks, it contained large areas of fatty tissue.

Analysis of the tissue contents of the interfragmentary gaps showed at one week after operation that there was little difference in tissue constituents between the two groups. Between three and 16 weeks in the healing group, there was an increase in bone whereas in the nonunion group the interfragmentary gap consisted predominantly of fibrous tissue (Fig. 6).

**Vascularity.** All the blood vessels were clearly stained as shown in Figure 7. The total number within the interfragmentary gap were counted for each time-point and the median values plotted in relation to time after operation (Fig. 8). Figure 9 shows the distribution of blood vessels between the intracortical and central regions of the interfragmentary gap. One week after operation there were significantly more blood vessels in the interfragmentary gap of animals in the non-stripped (healing) group (median 81, CI 14 to 138) than in those in the stripped (nonunion) group (median 10, CI 0 to 56). At one week, the blood vessels were present predominantly in the intracortical zone and there were significantly more blood vessels in the intracortical zone of the healing group than in the nonunion group. In the stripped group three weeks after surgery, the number of blood vessels distributed throughout the nonunion gap in both the central and intracortical regions, had increased significantly compared with that at one week. There was no statistical difference between the groups at three weeks. At eight weeks after operation there were significantly fewer blood vessels in the healing group (median 16, CI 10 to 20) compared with the nonunion group (median 136, CI 19 to 233). By 16 weeks after surgery the number of blood vessels in the healing group had decreased significantly and the number of blood vessels in the stripped group (median 84, CI 4 to 172) was higher than in the non-stripped group (median 5, CI 0 to 34). At eight weeks after surgery, the number of vessels in established atrophic nonunion had reached the same level as that during normal healing.
Photomicrographs at eight weeks showing a) in the stripped group the bone-ends which appear to be rounded and the interfragmentary gap filled with fibrous tissue (x 40) which b) appeared to be very vascular (inset from Fig. 5a), and c) in the non-stripped group formation of full bridging callus (x 20) with d) evidence of original lamellar bone (O) surrounded by new woven (W) bone (x 100).

Comparison of the mean proportions of tissue constituents (%) of the non-stripped and stripped (nonunion) groups at four different time-points after surgery.

Photomicrograph showing blood vessels within the interfragmentary gap clearly stained by anti-smooth muscle actin antibody (x 100).
**Discussion**

We have examined the density of blood vessels and the morphological characteristics during normal and impaired healing in a new experimental model of atrophic nonunion at early and late time-points.

One week after fracture, there was already a clear histological difference between the stripped and non-stripped groups. There was formation of periosteal callus in the non-stripped group and the interfragmentary tissue was more cellular at one and three weeks after operation. Three weeks after fracture, the interfragmentary tissue of the stripped group had become more cellular and in many animals there was evidence of muscle fibres infiltrating the gap. Previous research has suggested that soft tissues surrounding a fracture may contribute to the healing process and that primitive cells arise from muscle tissue.15,16

Three weeks after stripping of the periosteum, there was evidence of osteoclastic bone resorption causing the rounding of bone-ends. Volpon4 also noted osteoclastic bone resorption in a canine model of nonunion with absent or scanty callus.

At eight and 16 weeks after operation there was a very clear difference between the two groups. The non-stripped group had reached complete bony union, with full bridging callus. The stripped group had formed clear atrophic non-union with rounded bone-ends and fibrous tissue present in the interfragmentary gap. The presence of fibrous tissue in the model of atrophic nonunion at eight weeks was similar to that seen in established clinical nonunion.17 By 16 weeks, however, a mixture of fibrous and fatty tissue was present in the nonunion group. This may be the result of lack of stimulation of stem cells to form fibroblasts, chondroblasts, or osteoblasts, which caused the formation of adipocyte-like cells.

Previous studies of the vascularity of nonunion assessed hypertrophic nonunion using either 85strontium-labelled microspheres, vascular injection or bone scintigraphic methods.4,5 One assessed vascularity in a rabbit model of atrophic nonunion at one, eight and 16 weeks by immunohistochemical labelling.13

Volpon4 developed a model of radial nonunion in dogs in which there was a segmental defect of 5 mm with peristeal stripping of 2 cm. The bone was left unsplinted and resulted in hypertrophic nonunion in 54% and atrophic nonunion in 46%. He noted that there was good vascularity in both forms of nonunion.

In a similar study, dos Santos Neto and Volpon5 produced radial nonunion in dogs by resecting 3 mm of bone and 1 cm of periosteum and using bone wax to seal the cut bone surfaces. Again, the bone was left unsplinted and resulted in hypertrophic nonunion in 85% and oligotrophic nonunion in 15%. Vascular injection showed an increase in the peristeal, epiphyseal and medullary blood supply surrounding the site of the osteotomy as compared with normal animals. The model cannot be considered to be clinically relevant, however, because of the sealing of bone-ends with bone wax which is known to inhibit formation of new bone.18

In order to assess the effect of peristeal stripping on the healing of segmental bone defects in rats, Utvag, Grundnes and Reikeraos19 made a segmental excision of 8 mm, stabilised the bone using an intramedullary pin and then stripped the peristeum. Blood flow was assessed using microspheres labelled with 85strontium. It was decreased in both the stripped and non-stripped groups from four to 12 weeks after surgery. At eight weeks, the flow of callus was significantly higher in the stripped than in the non-stripped group.

Brownlow13 assessed vascularity in a rabbit model of atrophic nonunion immunohistochemically. The results were consistent with our findings with the exception that, at one week, there were no blood vessels present; in our study there was evidence of a small number of blood vessels. At eight weeks, there were more blood vessels present in the nonunion than in the control group, but this difference was
not statistically significant. A control group was not included at 16 weeks.

We assessed the number of blood vessels in a clinically relevant model of nonunion and showed that in atrophic nonunion the number reaches the same level as that in fractures which unite, but at a later time-point. One week after the operation there were significantly more blood vessels in the interfragmentary gap of the non-stripped group than in the stripped group. At three weeks, the number of blood vessels in the stripped group reached that of the non-stripped group at one week. At eight and 16 weeks, it reached that of the non-stripped group at three weeks. Thus, the atrophic nonunion reaches the same level of vascularity as that of a healing fracture, but at a later time-point. This, therefore, supports our hypothesis.

One week after surgery in the non-stripped group and three weeks after surgery in the stripped group, blood vessels were present predominantly in the periosteal and intracortical zones with few in the central zone. Similar findings were recorded by Brueton, Brookes and Heatley who carried out angiographic studies of osteotomies in rabbits and noted that the initial blood supply to the interfragmentary gap was periosteal. These results suggest that, when the periosteum is stripped, the blood supply recovers by three weeks after operation, with the blood vessels being present predominantly within the intracortical region. By three weeks in the non-stripped group, blood vessels are also present in the central zone. At eight weeks after operation the blood vessels are evenly distributed across the interfragmentary gap in the stripped group.

These results may explain some of the apparently conflicting views concerning the vascularity of atrophic nonunion. It has long been considered that insufficient vascularity is the predominant cause of atrophic nonunion, but many studies have shown good vascularity in established atrophic nonunion tissue. Diminished vascularity in the interfragmentary gap in the early stages of the repair of a fracture may prevent union. Our findings suggest that even when the interfragmentary gap becomes well vascularised at a later time-point this does not allow healing to occur and the atrophic nonunion persists.

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References