BASIC RESEARCH

Microbubble destruction with ultrasound augments neovascularisation by bone marrow cell transplantation in rat hind limb ischaemia

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Objective: To examine the effects of microbubble destruction with ultrasound (MB) combined with bone marrow derived mononuclear cell transplantation (BMT) into ischaemic tissues in rat hind limb ischaemia.
Methods and results: Unilateral hind limb ischaemia was surgically induced in Lewis rats. At postoperative day 7, rats were randomly divided into three groups: a vehicle treated group, an ultrasound treated group, and an MB treated group. MB treatment increased vascular endothelial growth factor mRNA as assessed by real time polymerase chain reaction (3.0-fold, $p < 0.05$). At four weeks, the MB group had increases in laser Doppler blood flow index (LDBFI; 1.2-fold, $p < 0.05$), angiographically detectable collateral vessels (angiographic score: 1.4-fold, $p < 0.01$), and capillary to muscle fibre ratio (1.5-fold, $p < 0.01$) in ischaemic limbs compared with the vehicle treated group. No differences were seen between the vehicle and ultrasound treated groups. Secondly, rats were allocated to vehicle treatment, BMT (5×10^6 cells/rat), or a combination of MB and BMT (MB+BMT) at seven days after hind limb ischaemia. BMT treatment significantly increased LDBFI, angiographic score, and capillary to muscle fibre ratio compared with vehicle treatment. Interestingly, MB+BMT treatment produced significantly greater LDBFI (1.2-fold, $p < 0.01$), angiographic score (1.5-fold, $p < 0.01$), and capillary to muscle fibre ratio (1.5-fold, $p < 0.05$) than BMT treatment alone.
Conclusions: MB may be a useful technique to enhance BMT induced neovascularisation.

Recently, investigators have begun to test the feasibility of using microbubbles for therapeutic purposes, primarily as targeted gene delivery systems.[1 2] These studies illustrate the promise of ultrasound–microbubble based therapeutics. Other studies have shown that the application of low frequency ultrasound to intravascular microbubble contrast agents creates small capillary ruptures.[3 4] Song *et al*[5] found that capillary rupture elicited by destruction of ultrasonic contrast agent microbubbles with 1 MHz ultrasound stimulated arteriolar remodelling in skeletal muscle. This remodelling response caused an increase in nutrient blood flow two weeks after treatment. Therefore, this technique may have the potential for restoring nutrient blood flow to skeletal muscle. However, the ultrasound–microbubble treatment was applied to normal rather than ischaemic hind limbs.

Endothelial progenitor cells (EPCs) have been found to participate in postnatal neovascularisation after mobilisation from bone marrow.[6] Previous studies have shown that bone marrow mononuclear cells (BM-MNCs) contain not only EPCs but also angiogenic factors and cytokines and that implantation of BM-MNCs into ischaemic tissues augments collateral vessel formation.[7 8] Several bone marrow subpopulations, such as EPCs and marrow derived stromal cells, may be able to differentiate into one or more of the cellular components of the vascular bed.[6 9] Kinnaird *et al*[10] showed that marrow derived stromal cells secrete a wide array of arteriogenic cytokines and can contribute to collateral remodelling through paracrine mechanisms. These studies suggested that BM-MNC transplantation (BMT) may be a useful strategy for therapeutic neovascularisation in ischaemic tissues in adults. However, techniques for enhancing BMT induced neovascularisation have not been well developed.

In the present study, we examined whether microbubble destruction with ultrasound (MB) augments neovascularisation in rat hind limb ischaemia and the effectiveness of microbubble treatment combined with BMT.

METHODS

Unilateral hind limb ischaemia and ultrasound application

The rat ischaemic hind limb model is a modification of a two stage procedure previously described.[11] Ischaemia was created in the left hind limb of inbred male Lewis rats (SLC, Shizuoka, Japan) under anaesthesia with sodium pentobarbital (50 mg/kg intraperitoneally). All left side branches of the aorta distal to the renal arteries and of the iliac artery were resected. On the same day as the second operation, the femoral artery was resected. In the first series of experiments, rats (n = 18) were subjected to unilateral hind limb ischaemia. At postoperative day 7, the control group (n = 6) and the second group (n = 6; ultrasound group) received a bolus intravenous injection of 0.3 ml saline through the jugular vein. The third group (n = 6; MB group) received a bolus intravenous injection of microbubble solution (0.3 ml of Optison; Nepa Gene Co Ltd, Chiba, Japan) in the same manner. Ten seconds after microbubble (or saline) injection, ultrasound treatment was applied to the ultrasound and MB groups with an ultrasound transducer

Abbreviations: BM-MNC, bone marrow mononuclear cells; BMT, bone marrow derived mononuclear cell transplantation; EPC, endothelial progenitor cell; LDBF, laser Doppler blood flow; LDBFI, laser Doppler blood flow index; MB, microbubble destruction with ultrasound; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor

(Sonitron 1000; Nepa Gene Co Ltd). A gracilis muscle was exposed to ultrasound for 10 minutes at 1 MHz, 2.0 W/cm^2 output intensity (corresponding to a mechanical index of 0.2), and 50% duty cycle. In the preliminary examination, acoustic destruction of microbubbles in the ischaemic hind limb was observed with an intravital charge coupled device camera microscope (RM-0001; Nihon Kohden, Tokyo, Japan). Microbubble (or saline) infusion and ultrasound treatment were applied three times a week until postoperative day 28.

Laser Doppler analysis and angiography

We measured the ratio of the ischaemic (left) to the normal (right) limb blood flow with a laser Doppler blood flow (LDBF) image analyser (Moor Instruments, Axminster, UK) as described previously.[12] Before laser scanning was initiated, rats were placed on a heating plate kept at 37℃ to minimise data variations due to body temperature. After blood flow was scanned twice, stored images were quantified by computer and the average flows of ischaemic and non-ischaemic limbs were calculated (moorLDI Image Processing version 3.08; Moor Instruments). To minimise variation due to ambient light and temperature, the LDBF index (LDBFI) was expressed as the ratio of ischaemic to non-ischaemic limb blood flow. The interobserver and intraobserver variabilities of LDBFI are 5.7% and 3.5%, respectively. Collateral formation was evaluated by using a Microfocus *x* ray television device (Hitex Co Ltd, Osaka, Japan) on day 28. A catheter was inserted through the right femoral artery and advanced to the lower abdominal aorta. Angiograms were taken two seconds after the injection of 0.5 ml contrast medium (Iopamiron; Schering). To quantitatively assess the extent of collateral vessel formation, we calculated the angiographic score as described previously.[13] The interobserver and intraobserver variabilities of the angiographic score are 6.3% and 4.5%, respectively.

Histological analysis

At day 30 (23 days after treatment), rats were killed with an overdose of pentobarbital. Four pieces of ischaemic tissue from the adductor and semimembranous muscles were obtained. Frozen sections were stained for alkaline phosphatase with an indoxyl-tetrazolium method to detect capillary endothelial cells as described previously.[13] Five fields from two muscle samples of each animal were randomly selected for capillary counts. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial oedema, the capillary to muscle fibre ratio was determined. For immunohistochemical analysis of the inflammatory responses and angiogenic factor, leucocyte infiltration was examined by immunohistochemical staining of CD45, a common leucocyte

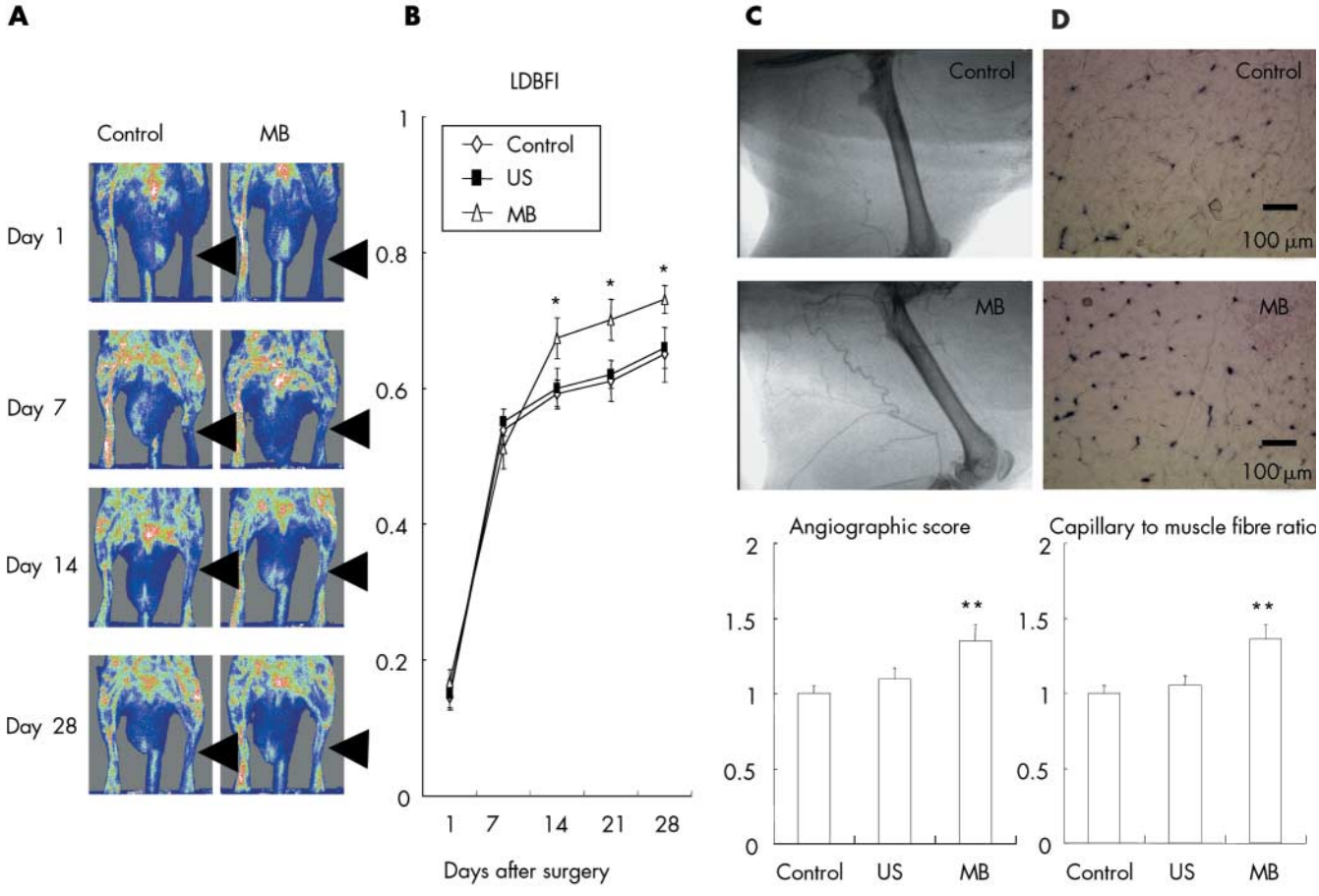

Figure 1 (A) Representative laser Doppler blood flow (LDBF) images. A low blood flow signal (dark blue) is observed in the ischaemic hind limb of the control group compared with a high blood flow signal (red to white) detected in the microbubble destruction with ultrasound (MB) group by LDBF. (B) Line graph of LDBF index (LDBFI) in each group. LDBFI was increased in the MB group compared with the control (n = 6 in each group). Ultrasound (US) alone did not change LDBFI compared with the control group. *p $<$ 0.05 *v* control. (C) Representative angiograms obtained on postoperative day 28. Numerous collateral vessels were observed in the MB compared with the control. Angiographic score of ischaemic hind limbs was significantly greater in the MB group than in the control. **p $<$ 0.01 *v* control. (D) Staining of ischaemic skeletal muscle tissues with alkaline phosphatase (dark blue) showing increased capillary counts in the MB group. Quantitative analysis showed significantly higher capillary to muscle fibre ratios in the MB group. **p $<$ 0.01 *v* control.

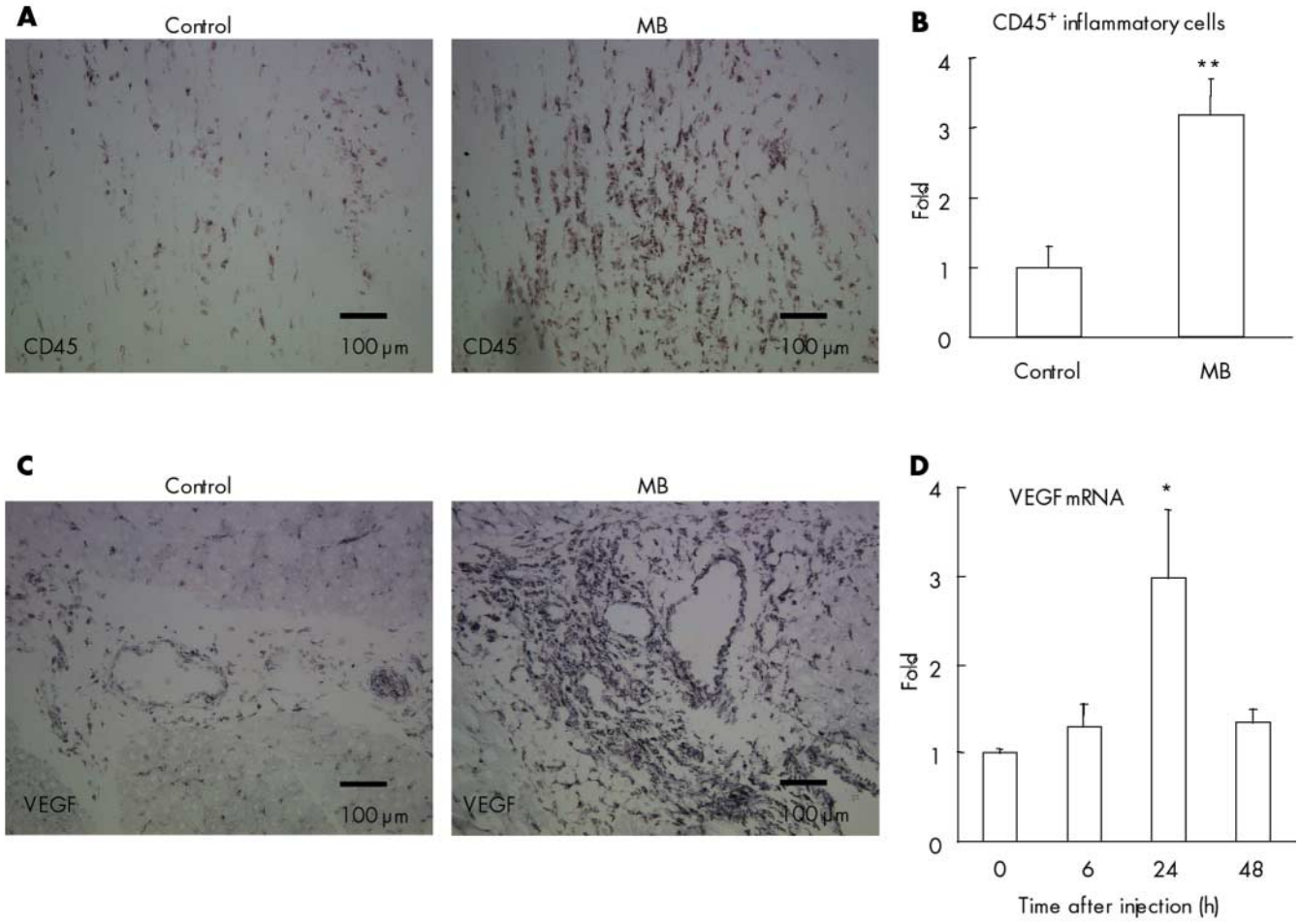

Figure 2 (A) Immunohistochemical staining for CD45 in ischaemic limbs 24 hours after US-microbubble treatment. Leucocyte infiltration was increased in the MB group compared with control. (B) Quantitative analysis showed increased CD45 positive inflammatory cell infiltration in the MB group compared with control. **$p < 0.01$ *v* control. (C) Immunohistochemical staining for vascular endothelial growth factor (VEGF) in ischaemic limbs 48 hours after US-microbubble treatment. Perivascular grey-black label corresponds to VEGF expression. VEGF expression was increased in the MB group compared with control. (D) Quantitative real time reverse transcription and polymerase chain reaction analysis. Data were obtained from six independent ischaemic muscles for each time point and are expressed as mean (SEM) of mRNA normalised to glyceraldehyde-3-phosphate dehydrogenase. *$p < 0.05$ *v* 0 hour.

antigen, and vascular endothelial growth factor (VEGF). Rats were killed two days after microbubble treatment. The ischaemic hind limbs were immediately embedded in optimal cutting compound (Tissue Tek, Miles Inc), frozen in dry ice-acetone, and cut into 5 µm sections with a cryostat. Either mouse anti-rat CD45 monoclonal antibody (BD Biosciences) or rabbit anti-human VEGF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used as the primary antibody and the secondary antibody was anti-mouse or anti-rabbit polyvalent-peroxidase conjugate (Nichirei, Japan). Peroxidase activity was visualised with 3,3′-diaminobenzidine as a chromogen.

VEGF mRNA quantification

To elucidate the effects of MB on the expression of angiogenic growth factors, we investigated VEGF mRNA expression in the ischaemic hind limb by real time reverse transcription polymerase chain reaction (PCR). Changes in the amount of VEGF mRNA in the ischaemic muscle were quantified by reverse transcription PCR. The rats were killed at predetermined arbitrary time points after microbubble treatment with an overdose of sodium pentobarbital. The total RNA was extracted from ischaemic tissue samples and reverse transcribed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences). The synthesised cDNA was quantified by TaqMan quantitative PCR analysis of each gene with the ABI PRISM 7700 detection system (Applied Biosystems, Foster City, California, USA) according to the manufacturer's protocol.

Isolation of rat BM-MNCs

We examined the effect of microbubble treatment combined with BMT. Bone marrow was harvested by flushing the tibias and femurs of Lewis rats with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine medium. The plug of whole marrow cells was dispersed by passing it through pipettes of decreasing sizes. After a homogeneous cell suspension was achieved, mononuclear cells were isolated by density gradient centrifugation (Nyco Prep 1.077 Animal; Axis-Shield PoC AS, Oslo, Norway).

Combination of microbubble treatment and BMT

Additional rats (n = 21) were subjected to unilateral hind limb ischaemia and randomly divided into three groups. At day 7, the control group (n = 7) received 2.5 ml saline. The second group (n = 7) received BM-MNCs (5×10^6 cells/animal; BMT group) transplanted into the ischaemic thigh skeletal muscle with a 26 gauge needle at six different points. The third group (n = 7) received microbubble treatment followed by BMT (5×10^6 cells/animal; MB+BMT group).

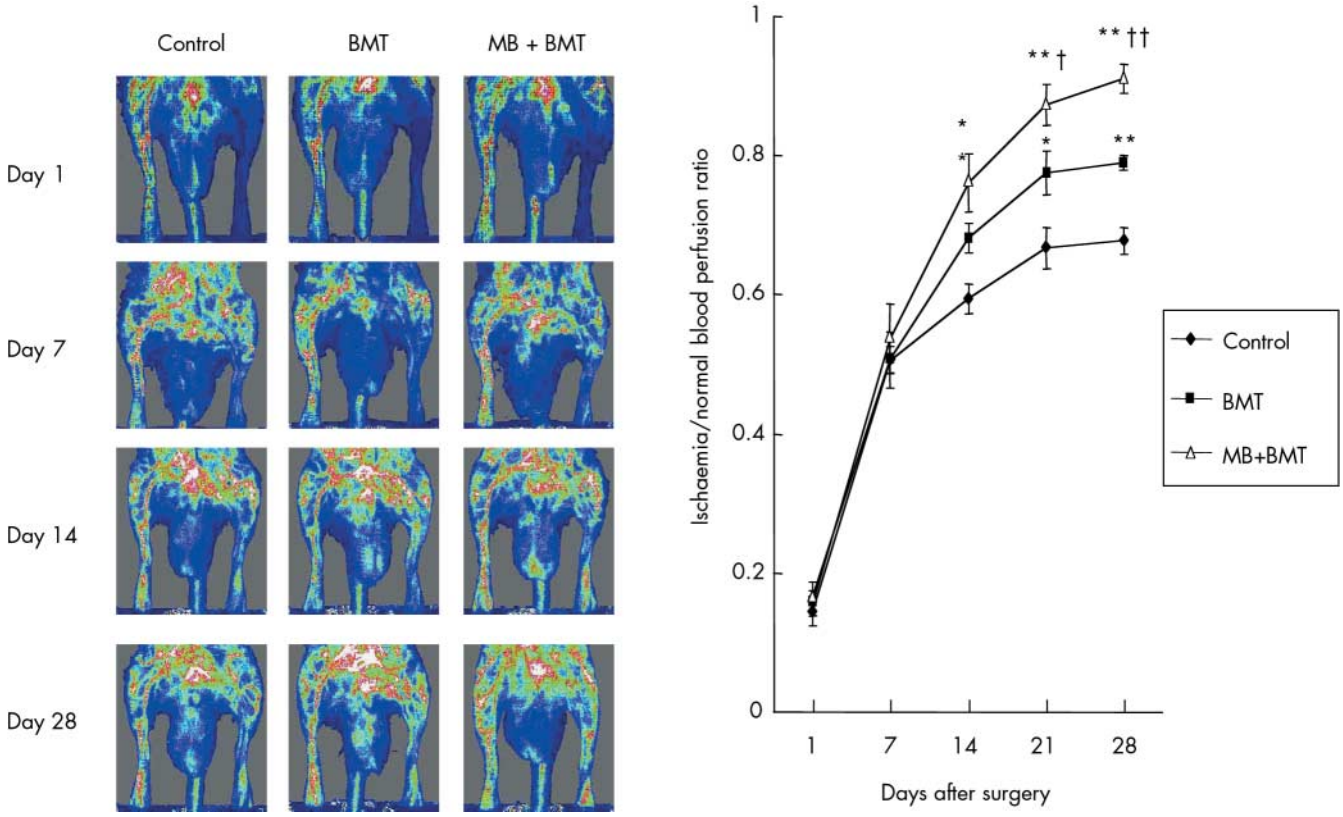

Figure 3 Representative LDBF images and line graph of calculated LDBFI. LDBFI increased significantly in the bone marrow mononuclear cell (BM-MNC) transplanted group (BMT) compared with control. The combination of microbubble treatment and BMT (MB+BMT) led to a significant further increase in LDBFI compared with BMT. *p < .05 *v* control; **p < 0.01 *v* control; †p < 0.05 *v* BMT; ††p < 0.01 *v* BMT.

Laser Doppler and histological analyses were performed as described above.

Statistical analysis

Results were expressed as mean (SEM). Significance was determined by analysis of variance and the Student-Newman-Keuls test. Differences were considered significant at $p < 0.05$.

RESULTS

Neovascularisation by MB

To analyse subcutaneous blood flow, LDBF was analysed once weekly after hind limb ischaemia (fig 1A, B). An increase in LDBFI was observed in the ischaemic limb of the MB group (1.2-fold increase at day 28, $p < 0.05$) compared with the control group. No significant differences in LDBFI were observed between the ultrasound group and the control group. At postoperative day 28, all animals were subjected to iliac angiography. Figure 1C shows representative angiograms. Collateral vessels were observed in the MB group rather than in the control group and the ultrasound group. Quantitative analysis by angiographic scores showed a significantly greater number of collateral vessels in ischaemic tissue from the MB group (1.4-fold increase, $p < 0.01$) than from the control group. Ultrasound treatment alone did not change the angiographic score compared with vehicle treatment. The medial thigh muscles of the ischaemic limbs were histologically examined at day 30 as described above. Figure 1D shows representative photomicrographs of histological sections from ischaemic tissue. Alkaline phosphatase staining showed the presence of numerous capillary endothelial cells in the MB group but a lower number of capillary endothelial cells in the control group. The capillary to muscle fibre ratio was greater (1.4-fold, $p < 0.01$) in the MB group than the control group. In contrast, the ratio of the ultrasound group did not differ significantly from that of the control group. Thus, the ultrasound treatment alone did not affect ischaemic tissue, whereas MB induced angiogenesis in this rat hind limb ischaemic model. [1]

Inflammatory responses and VEGF expression in ischaemic limbs after MB

CD45 immunostaining showed major infiltration of inflammatory leucocytes in the MB group (fig 2A). The number of CD45 positive leucocytes was higher in the MB group than in control (fig 2B). Immunohistochemical staining with anti-VEGF showed a higher level of VEGF expression in the perivascular tissues retrieved from microbubble treated rats than in controls at day 2 after treatment (fig 2C). Moreover, VEGF mRNA expression was significantly increased (3.0-fold, $p < 0.05$) at 24 hours after ultrasound–microbubble treatment compared with the level before treatment (fig 2D).

MB augments neovascularisation by transplanted BM-MNCs

We investigated the effects of ultrasound–microbubble treatment in combination with BMT on neovascularisation in hind limb ischaemia. Hind limb blood flow was serially examined by LDBF analysis once weekly after surgery (fig 3). The LDBFI was significantly increased in the BMT group compared with the control group at days 21 and 28. Combination treatment (MB+BMT) led to a significant further increase in the LDBFI compared with BMT monotherapy at days 21 and 28. Formation of collateral vessels was evaluated by angiography at postoperative day 28 (fig 4A, B). Monotherapy with BMT increased the angiographic scores significantly compared with control and MB+BMT improved the angiographic scores further. To further evaluate the effect of BMT and MB+BMT on neovascularisation, the ischaemic

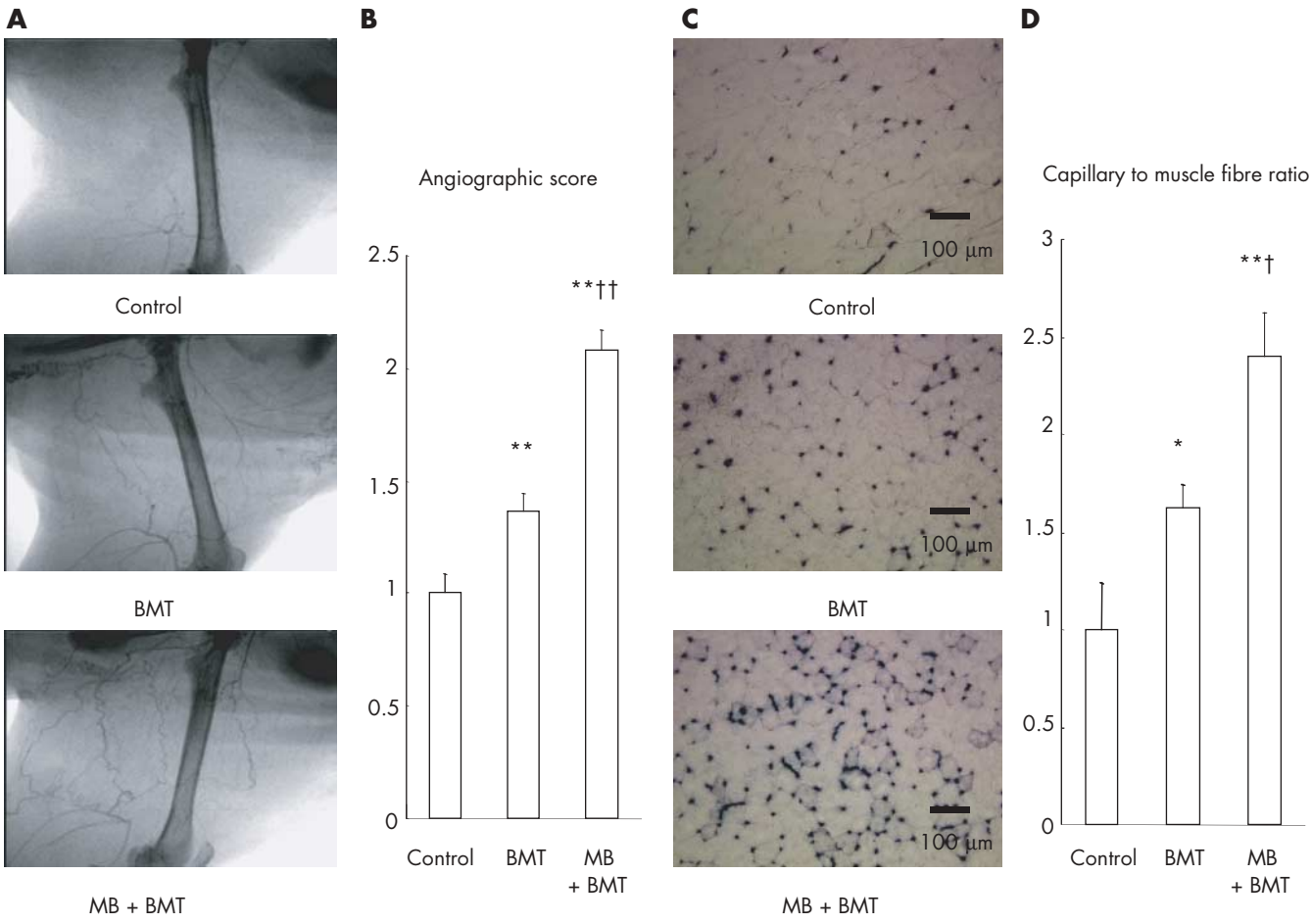

Figure 4 (A) Representative angiograms obtained on postoperative day 28. Numerous collateral vessels developed in the BMT and MB+BMT groups but not in controls. (B) Angiographic scores in ischaemic hind limbs were significantly higher after BMT than in controls. MB+BMT significantly further increased angiographic scores compared with BMT. **p < 0.01 *v* control; ††p < 0.01 *v* BMT. (C) Staining of ischaemic skeletal muscle tissues with alkaline phosphatase (dark blue) showing increased capillary count in the BMT and MB+BMT groups. (D) Quantitative analysis showed significantly higher capillary to muscle fibre ratios after BMT than in controls. MB+BMT led to a significant further increase in capillary to muscle fibre ratio compared with BMT. *p < 0.05 *v* control; **p < 0.01 *v* control; †p < 0.05 *v* BMT.

muscles were examined histologically at day 30 as described above (fig 4C, D). BMT significantly increased the capillary to muscle fibre ratio compared with control. Moreover, MB+BMT led to a further increase in capillary to muscle fibre ratio compared with BMT.

DISCUSSION

The major findings in the present study are, firstly, that MB induced neovascularisation in the ischaemic hind limb; and secondly, that ultrasound–microbubble treatment augmented neovascularisation by transplanted BM-MNCs in the ischaemic hind limb.

Although a previous study had suggested that inflammation created by the rupturing of capillaries initiates the arteriogenesis response,[5] the exact mechanism by which arteriogenesis is stimulated remains to be determined. Given the abundance of evidence that transmyocardial laser revascularisation induces neovascularisation through wound healing responses,[14 15] it is suggested that these small capillary ruptures may elicit neovascularisation through a similar mechanism. Recent studies have shown that transmyocardial revascularisation is associated with a significant angiogenic response, which appears to be mediated by the release of angiogenic growth factors such as VEGF, basic fibroblast growth factor, and transforming growth factor β.[16 17] In the present study, we observed that VEGF was upregulated after ultrasound–microbubble treatment in ischaemic muscle, suggesting a role for VEGF in ultrasound–microbubble treatment induced neovascularisation.

MB induces vascular injury. The repair of injury begins with the release of peptide growth factors from both inflammatory cells and injured cells as soon as tissue is damaged. Leucocytes affect many angiogenic processes, in part because leucocyte subtypes produce a large number of angiogenic factors, including VEGF, basic fibroblast growth factor, and various interleukins and proteinases (tryptase, chymase, matrix metalloproteinases, heparanase, and urokinase plasminogen activator).[18] Furthermore, Mohle *et al*[19] showed that thrombin activated platelets release VEGF and they suggested that VEGF delivered to sites of vascular injury by activated platelets may initiate angiogenesis. Taking together these results and our findings, it is reasonable to postulate that accumulation of blood cells after microbubble destruction elicits neovascularisation by supplying angiogenic factors.

Neovascularisation by VEGF in vivo[13] has been attributed to its mitogenic and promigratory effects on endothelial cells,[20 21] consistent with the concept that angiogenesis occurs by way of the development of sprouts from pre-existing, fully differentiated endothelial cells. More recent studies have shown that VEGF contributes to postnatal neovascularisation by mobilising bone marrow derived EPCs.[22] It is suggested that VEGF may facilitate tissue neovascularisation, pre-

viously attributed exclusively to angiogenesis, in part by mobilising EPCs to contribute to vasculogenesis.

Furthermore, direct effects of VEGF on transplanted BM-MNCs probably contributed to improved limb neovascularisation. VEGF is known to be essential for the in vitro differentiation of purified EPCs into mature endothelial cells.[23] This observation is consistent with in vivo studies showing the importance of VEGF for vasculogenesis.[24] Given that EPCs by definition express VEGF receptors, VEGF may also enhance EPC proliferation, adhesion, incorporation into endothelial cell monolayers, and differentiation to endothelial cells as reported previously.[24–26] These studies show the pivotal importance of VEGF and its receptors in blood vessel development. In the present study, BMT augmented angiogenesis and collateral vessel formation in ischaemic tissue. MB enhanced neovascularisation by transplanted BM-MNCs. In addition to the mechanical effects of capillary rupture by microbubble destruction, the augmentation may be partially due to VEGF expression after microbubble destruction. Altogether, local overexpression of VEGF and EPCs may promote neovascularisation in the target ischaemic tissues.

On the other hand, it has been reported that ultrasound can induce oxidative stress, endothelial cell damage, and increased microvascular permeability.[27 28] MB induced an inflammatory response causing local leucocyte infiltration, which may induce apoptosis and loss of cell membrane integrity.[29 30] Because the angiogenic effects may be related to tissue damage, further studies are needed to elucidate the clinical safety of this method.

In summary, our results show that MB can augment neovascularisation by transplanted BM-MNCs in the ischaemic hind limb. Microbubble treatment is likely to induce collateral vessel formation partly by supplying VEGF. The ultrasound-Optison method may be useful as a clinical intervention to enhance neovascularisation after BMT.

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