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Therapeutic Angiogenesis/Arteriogenesis in the Chronic Ischemic Rabbit Hindlimb: Effect of Venous Basic Fibroblast Growth Factor Retroinfusion

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Therapeutic induction of angiogenesis has been shown in experimental hindlimb ischemia. An alternative to targeting the ischemic hindlimb tissue via the severely stenosed or occluded artery consists in the intact venous system, e.g., by retroinfusion. We tested whether basic fibroblast growth factor (bFGF) enhances angiogenesis induction. Therefore, we applied bFGF retrogradely as compared to intramuscular application. Furthermore, we assessed whether bFGF-induced angiogenesis was enhanced by low-dose VEGF coapplication. Chronic hindlimb ischemia in rabbits was established by excision of the femoral artery at day 0 (d0). At d7, baseline collateral number in the ischemic limb and collateral flow velocity of contrast agent (frame count score) were assessed. Thereafter, saline solution (control group) or bFGF (20 μg/kg) with or without VEGF (10 μg/kg) was retroinfused through the femoral vein. Alternatively, bFGF (20 μg/kg) was injected into thigh and calf muscles. At d35, collateral growth and flow velocity were quantified, and tissue samples were analyzed for capillary density. In the untreated control group, capillary/muscle fiber (C/FM) ratio of the ischemic limb was 0.87 ± 0.12, and collateral number as well as frame count score at $-$d35 did not change compared to d7 ($107\% \pm 7\%$ and $109\% \pm 10\%$ of baseline, respectively). Retrograde application of bFGF induced capillary and collateral growth (C/FM ratio 1.56 ± 0.19 and frame count $161\% \pm 29\%$ of baseline), resulting in enhanced flow velocity ($143\% \pm 13\%$), similar to the intramuscular application of bFGF. Additional low-dose VEGF retroinfusion did not further increase capillary/collateral growth (1.49 ± 0.08 and $172\% \pm 26\%$) nor perfusion velocity ($149\% \pm 7\%$). The authors conclude that bFGF retroinfusion is a feasible approach of inducing angiogenesis and arteriogenesis in an ischemic hindlimb, resulting in increased blood perfusion, which was not further extended by additional low-dose VEGF coapplication.

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Patients with hypoperfusion of the ileo-femoro-popliteal arteries are prone to develop ischemic leg syndromes, including claudication, inability to move, ischemic ulcers, and gangrene. Therapeutic neovascularization is a novel approach for treatment of this condition. Neovascularization is a complex process, comprising angiogenesis and arteriogenesis as two distinct processes typically found in adult organisms. Both entities are different from vasculogenesis, de novo vessel formation achieved by pluripotent stem cells, notably during embryonic development, which also occurs in the adult organism (Luttun and Carmeliet 2003). Angiogenesis (Isner et al. 1996), defined as capillary growth by sprouting from enlarged venules, is distinguished from arteriogenesis or enlargement of preexisting collaterals, essentially involving smooth muscle cell growth (Schaper and Buschmann 1999). The conceptual separation of both processes is not realized in vivo, because, for example a single bolus of a recombinant growth factor such as vascular endothelial

growth factor (VEGF)-C induces angiogenesis and arteriogenesis in vivo (Witzenbichler et al. 1998).

Importantly for VEGF-A, therapeutic doses (0.5 to 1 mg per animal) were found to induce severe hypotension (Sato et al. 2001). The latter side effect of VEGF-A, associated with accelerated limb loss in mice (Masaki et al. 2002), is at least in part due to increased nitric oxide (NO) formation (Sato et al. 2001) by phosphorylation of endothelial NO synthase (eNOS) (Fulton et al. 1999; Dimmeler et al. 1999). Moreover, high local concentrations of VEGF-A are capable of hemangioma induction in murine limbs and hearts (Schwarz et al. 2000; Lee et al. 2000; Springer et al. 1998).

A growth factor offering a more favorable pharmacologic profile is basic fibroblast growth factor (bFGF, FGF-2), which also has displayed proangiogenic (Watanabe et al. 1998) and proarteriogenic (Deindl et al. 2003; Wempe et al. 1997) properties. Acute eNOS activation has not been described in cell culture (Ziche et al. 1997), and hypotensive effects have been experienced at submaximal doses in man (>30 μg/kg) (Simons et al. 2002). Interestingly, a proangiogenic synergism of bFGF (10 μg) and VEGF-A (500 μg) was noted in ischemic rabbit hindlimbs in vivo (Asahara et al. 1995), using, however, a VEGF-A dose at risk of inducing hypotension (Sato et al. 2001).

Therefore, the purpose of the present study was to investigate the neovascularization potential of bFGF (20 μg/kg), with or without low dose VEGF-A (10 μg/kg). In the same study, we tested the efficacy of local retrograde intravenous application of bFGF in comparison to intramuscular application.

MATERIALS AND METHODS

New Zealand rabbits (3 to 3.5 kg, male) were purchased at Charles River Germany (Sulzfeld, Germany). Rompun was from Bayer (Leverkusen, Germany) and ketamine was purchased from Ratiopharm (Ulm, Germany). bFGF and VEGF were from PeproTech (Frankfurt, Germany), and the contrast agent Solutrast 370 was provided by Byk Gulden (Konstanz, Germany).

Animal Protocol

All animal experiments were approved by the Bavarian Animal Care and Use Committee (AZ 211-2531-58/99 and AZ 211-2531-82/02).

At day 0, after intravenous (i.v.) anesthesia induction with rompun (2 mg/kg) and ketamine (50 mg/kg), the right femoral artery was surgically prepared. All side branches from the origin of the external iliac artery to the bifurction of saphenous and popliteal arteries were ligated with 4.0 silk and the complete femoral artery was excised (Naya et al. 2002). The wound was closed and animals were brought back to the animal facility with free access to food and water.

At day 7, animals were anesthetized as described above, and a baseline angiography was performed after positioning a 4-French catheter (Cordis, Haan, Germany) via the right carotid artery into the infrarenal portion of the abdominal aorta. Contrast agent was injected into the ischemic limb with an automatic injector (2 ml/s, 4 ml total) and fluoroscopy was performed with a Siemens system (Munich, Germany). Twenty-five angiographic pictures (frames) per second were acquired and stored for later analysis (ACOM, Siemens, Germany).

Pressure-Controlled Retroinfusion of the Ischemic Hind Limb

The femoral vein was surgically prepared and a catheter (1.22 mm outer diameter) was inserted. A cuff was placed proximal to the femoral vein around the ischemic limb, and fluoroscopic control of venous outflow occlusion was performed. Thereafter, retroinfusion of heparinized sodium chloride 0.9% (10 ml) without growth factors (control group), with bFGF (20 μg/kg) or with combined bFGF (20 μg/kg) and VEGF (10 μg/kg) was performed over 30 min. Pressure was continuously monitored and maintained at 80 mm Hg, a level sufficient to allow complete filling of veins (Schoop and Acevedo 1993). Retroinfusion pressure did not exceed 100 mm Hg, with no venous rupture observed throughout the experimental series.

Using ^{99m}Tc-nanocolloids, a 5.8-fold increase of specific radioactivity in the ischemic limb was found 30 min after retrograde as compared to the antegrade application. The prolonged retention of the radiotracer after reopening of the cuff is consistent with extravasation of small molecules into the interstital space (see Fig. 1). At day 35, after anesthesia application as described above, a second fluoroscopy of the ischemic hindlimb was performed with motorpump infusion of contrast agent (4 ml/2 s), providing data for collateral score and frame count score.

Capillary Density

At the end of the experiment, tissue samples from the thigh (m. adductor) and calf muscles (m. gastrocnemius, m. tibialis anterior) were obtained for quantification of capillary density. Transverse 7-mm sections were cut from each muscle specimen using a cryostat (Leitz, Wetzlar, Germany). For detection of capillary endothelial cells, sections were routinely stained for alkaline phosphatase or for confirmation with a CD31 antibody (Santa Cruz SC1506) and a fluorescein isothiocyanate (FITC)-labeled secondary antibody (cf. Fig. 2*A*). Capillaries and myofibers were counted in each microscopic field (40× magnification) in a blinded fashion, capillary density being expressed as capillary:myofiber ratio as described earlier (Brown and Hudlicka 1988).

Collateral Score

Angiographies were analyzed for collaterals by counting the number of vessels in the femoral region (from the iliac bifurcation to the popliteal bifurcation) intersecting an overlaying grid, as described previously (Arras et al. 1998). The score obtained at day 35 (e.g., Fig. 3*A–C*) was normalized to baseline score obtained at day 7.

Frame Count Cinedensitometry

Quantitative measurement of blood flow by assessing the time a contrast agent requires for passage between two defined

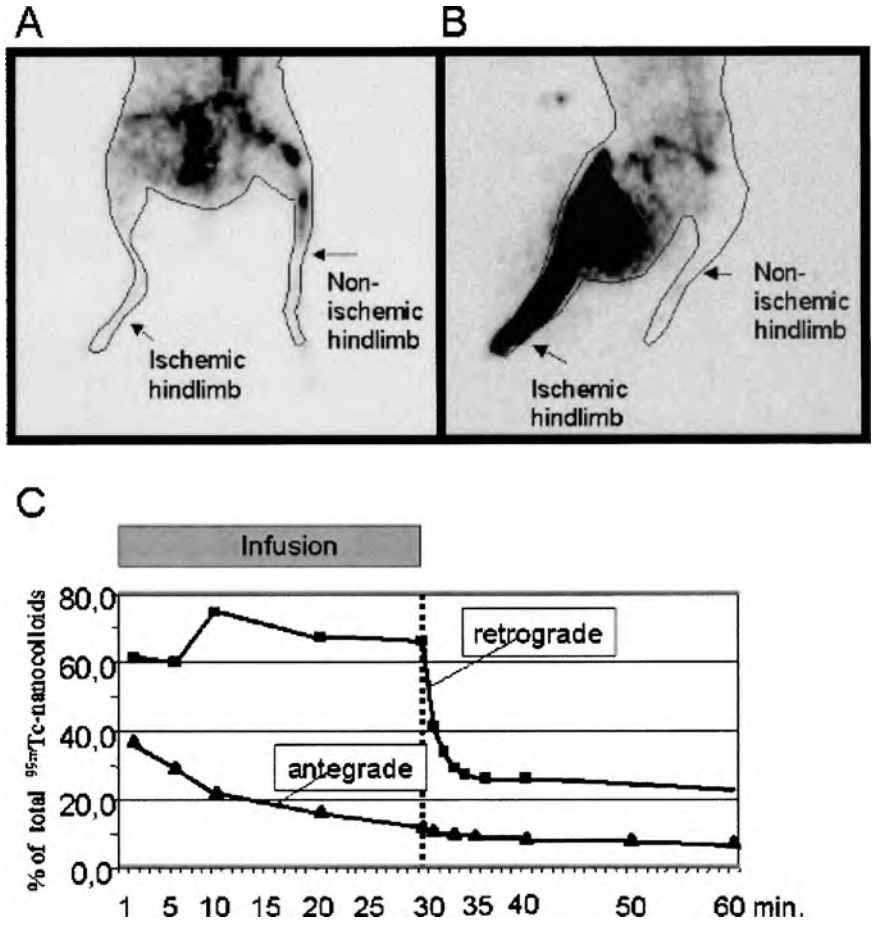

FIG. 1. *A*, Example of a rabbit hindlimb 7 days after excision of the femoral artery, after intra-arterial infusion of ^{99m}Tc-nanocolloids (100 MBq). *B*, A parallel experiment of an ischemic rabbit hindlimb after retrograde intravenous infusion of ^{99m}TC-nanocolloids (100 MBq). *C*, Quantitative analysis of the distribution of ^{99m}TC-nanocolloids revealed a 5.8-fold increase of specific radioactivity immediately after retrograde as compared to antegrade delivery. Thirty minutes later, the increase was still 2.8-fold (data are given in percent of total detectable radioactivity [whole body region of interest]).

anatomical landmarks has been established in femoral, iliac, and coronary arteries (Swanson et al. 1983; Gibson et al. 1996; Dorsaz et al. 1997). In the present investigation, we used cineangiograms obtained as described above, defining the landmarks as the proximal and distal end of the excised femoral artery, i.e., the bifurcation of internal and external iliac arteries and the proximal popliteal artery. Peak densitometry at the proximal and distal landmark was determined and the number of frames required for peak densitometry to pass from the proximal to the distal landmark were counted (cf. Fig. 4). Because blood flow velocity is indirectly proportional to frame count, and frame count in the ischemic limb (i.l.) was normalized to the nonischemic contralateral limb (n.l.), we assessed blood flow with the following formula:

$$\text{Frame count score} = \frac{1/\text{frame count (i.l.)}}{1/\text{frame count (n.l.)}}$$

$$\text{Frame count score} = \frac{\text{frame count (n.l.)}}{\text{frame count (i.l.)}}$$

The changes in frame count score from days 7 to 35 are given in percent of day 7. This collateral flow analysis correlated well with other established perfusion measurements, e.g., flow wire measurement in the internal iliac artery of the ischemic limb (Fig. 4*C*).

Microsphere Measurement

For further confirmation of the validity of blood flow velocity analysis via frame count assessment in the present model, microsphere injections were conducted as reference measurements for blood flow in the ischemic hind limb in control and bFGF/VEGF-treated animals. A catheter was placed in the left ventricle and microspheres were injected over 30 s into the ventricle. Arterial reference blood samples were drawn at a fixed rate to assess distribution of microspheres in the circulation. At the end of the experiment, probes were taken from thigh and calf muscles to assess blood flow in the upper and lower regions of the ischemic hind limb and the nonischemic contralateral limb. Probes were lysed, as previously described (Boekstegers et al.

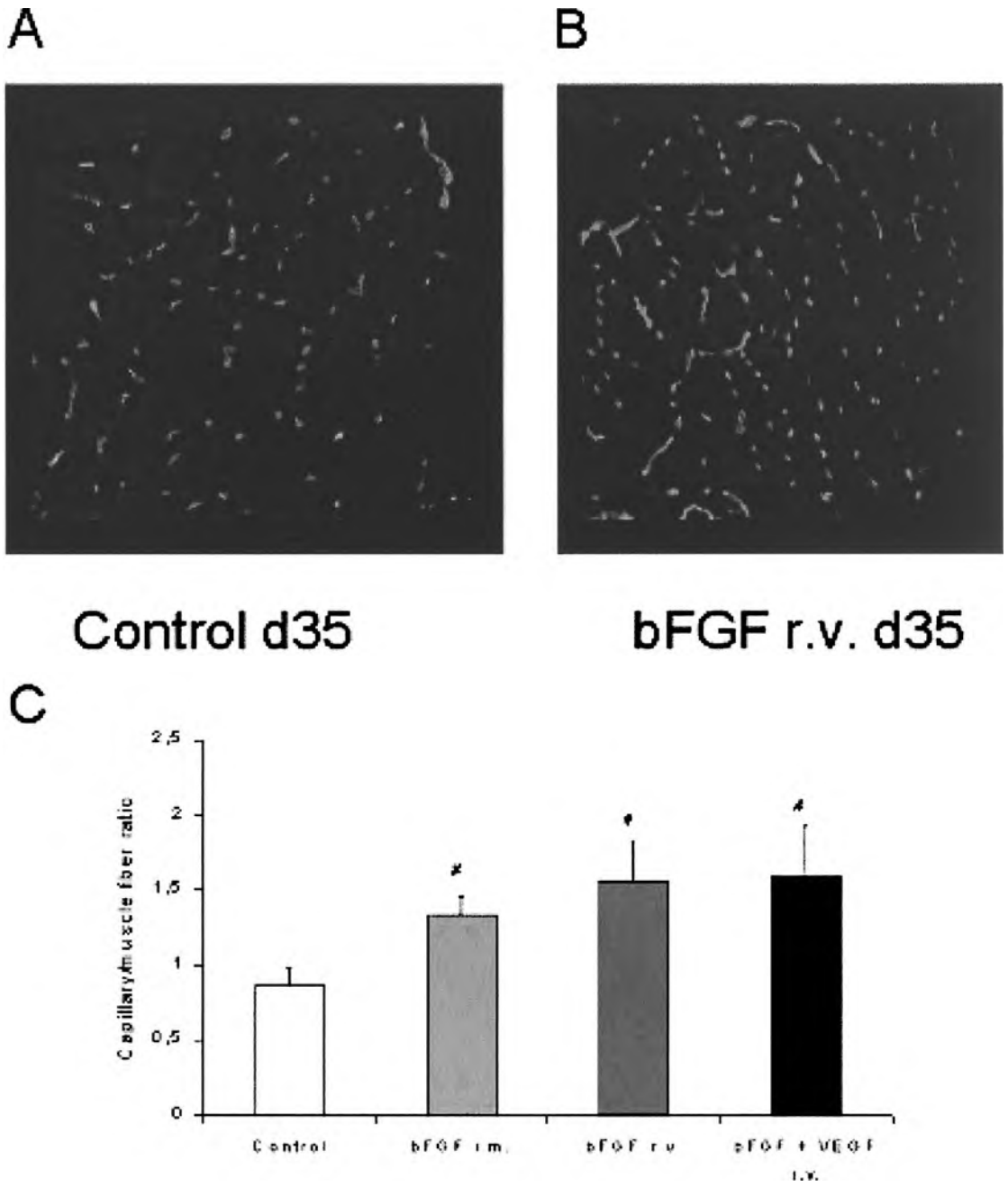

FIG. 2. Capillary:muscle fiber ratio (PECAM-1 staining) of the *M. tibialis* anterior of a hindlimb retroinfused with saline (*A*) or bFGF (*B*). *C*, Quantitative analysis revealed an increase of capillary:muscle fiber ratio after i.m. (intramuscular) and r.v. (retrograde) bFGF application and bFGF + VEGF retroinfusion. $n = 6$, $^{\#}p < .05$ versus saline-retroinfused controls.

2002), and microsphere fluorescence was quantified in a Perkin Elmer spectrometer (Fig. 4*D*).

Statistical Analysis

Data are given as mean ± *SEM* for $n = 6$ animals per group. Differences between several groups were tested using analysis of variance (ANOVA) and Student Newman Keul's post hoc analysis; $p < .05$ was considered statistically significant.

RESULTS

Capillary Density

In saline retroinfused control experiments, capillary/muscle fiber (C/FM) ratio of the ischemic limb was similar to the contralateral limb (0.87 ± 0.12 versus 0.81 ± 0.05). However, bFGF retroinfusion induced a significant increase in C/FM ratio (1.56 ± 0.19), similar to intramuscular bFGF injection (1.33 ± 0.13). Co-retroinfusion of bFGF and low-dose VEGF

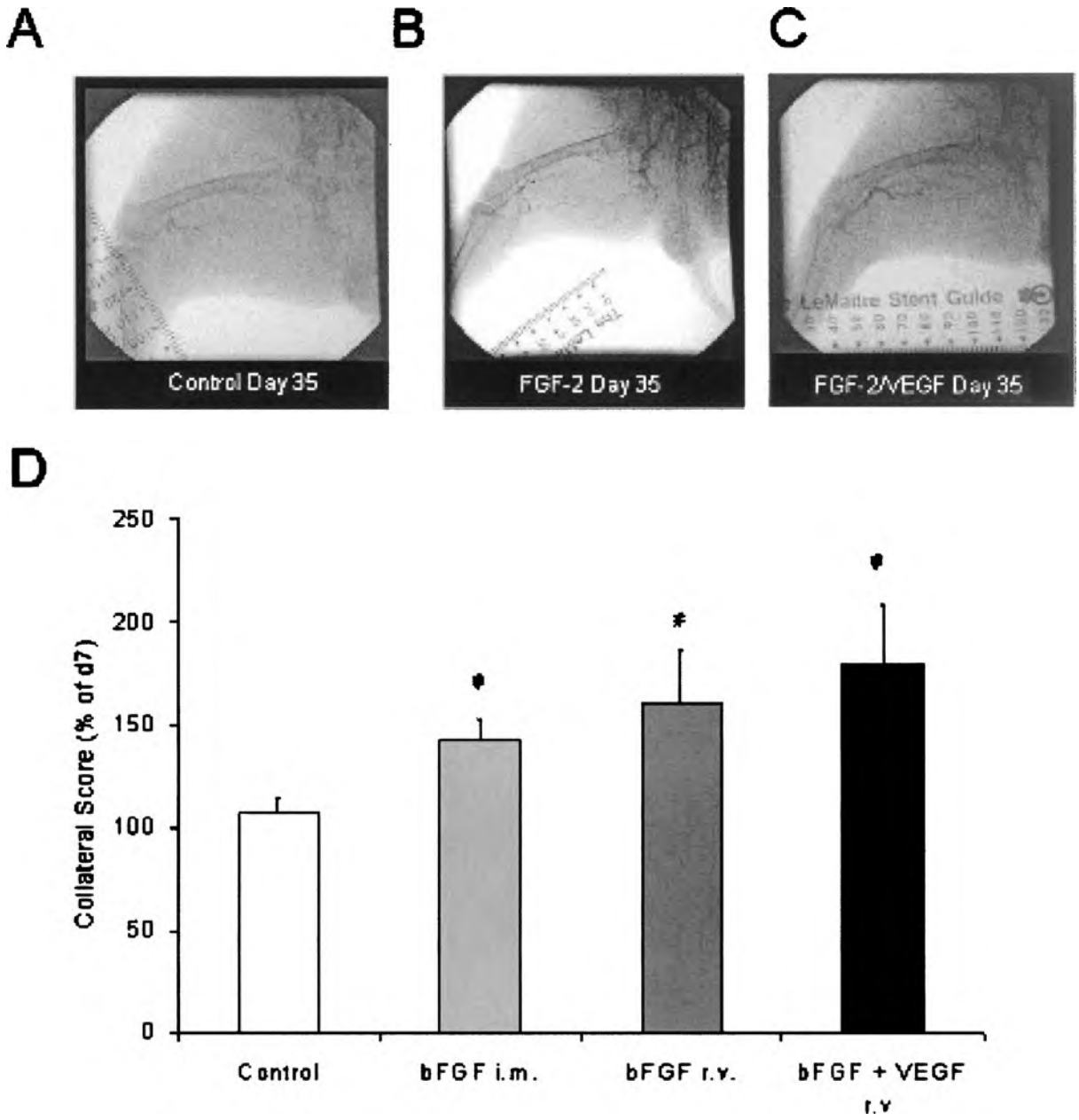

FIG. 3. Collateral score in ischemic rabbit hindlimbs, quantified as number of vessels intersecting an overlaying grid. *A*, saline retroinfusion; *B*, bFGF intramuscular application; *C*, bFGF retroinfusion; *D*, quantitative analysis of collateral growth (cf. Materials and Methods) at day 35. $n = 6$, $^{\#}p < .05$ versus saline-retroinfused controls.

did not further increase C/FM ratio compared to bFGF application alone (1.49 ± 0.08).

Collateral Score

In saline-retroinfused controls, the collateral score at day 35 did not significantly change as compared to day 7 (34 ± 5 compared to 30 ± 3, $107\% \pm 7\%$) (see Fig. 3*C*). bFGF retroinfusion increased the collateral score to $161\% \pm 29\%$, a level not significantly altered by additional VEGF retroinfusion ($172\% \pm 26\%$). The increase in collateral score after intramuscular bFGF treatment was comparable to that of the retroinfused group ($142\% \pm 14\%$). Thus, bFGF treatment either by retroinfusion or by intramuscular injection induced arteriogenesis, a feature not significantly augmented by low-dose VEGF coapplication.

Blood Flow Velocity

Next we asked whether the detected increase in capillary density and collateral flow would serve to increase blood flow to the ischemic limb. To assess blood flow, frame count analysis was established using the fluorscopic pictures (cf. Materials and Methods).

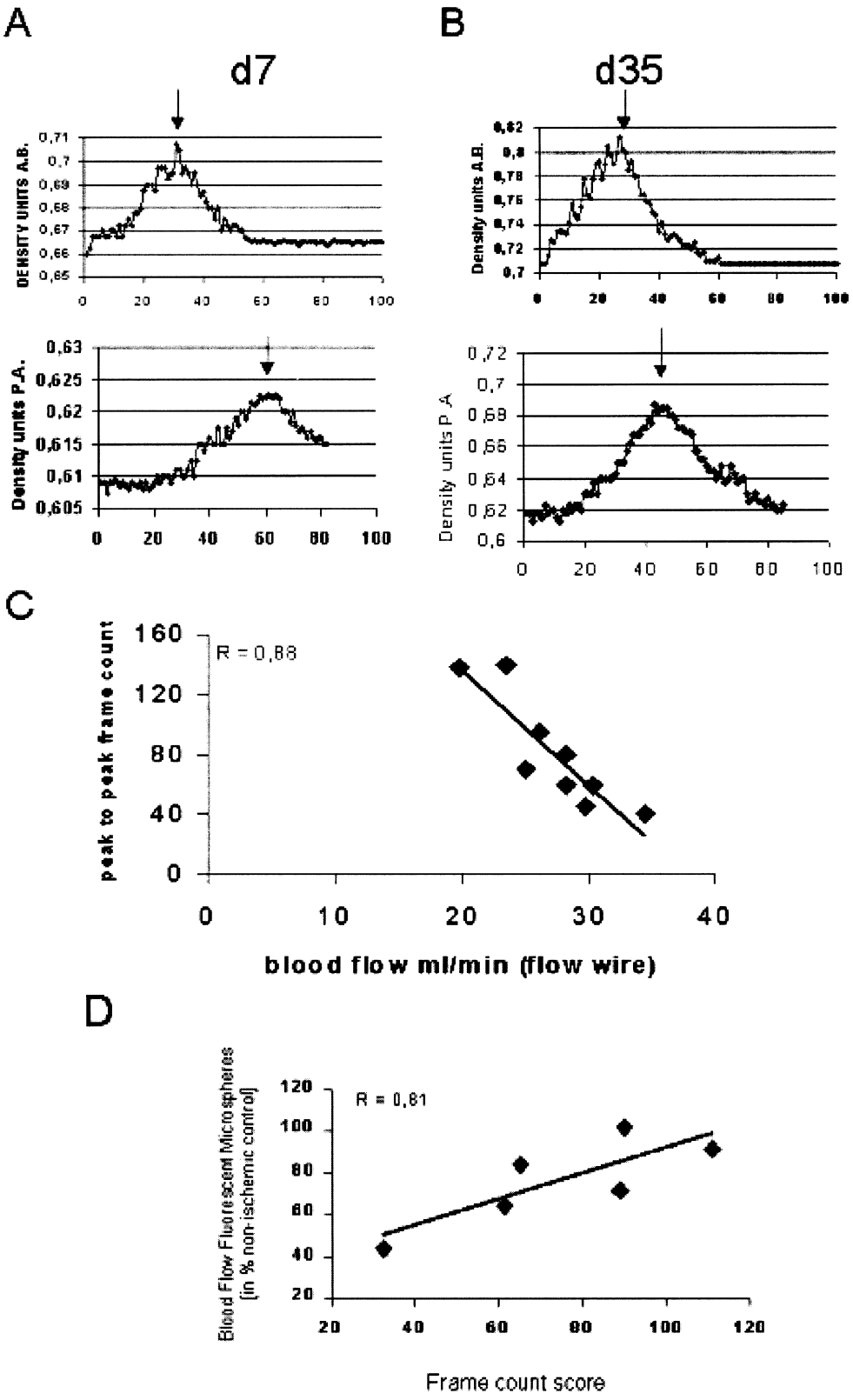

FIG. 4. *A*, *B*, Determination of blood flow via cinedensitometry. Example of rabbit hindlimb with retrograde infusion of bFGF. Compared to baseline measurements at day 7 (*left panels*), at day 35 (*right panels*) the number of cine frames counted from peak density of contrast agent at the aortic bifurcation (A.B.) to peak density at popliteal artery (P.A.) is reduced. *C*, Correlation of cinedensitometric measurements and flow wire analysis. The frame count correlated inversely with the blood flow assessed by flow wire inserted in the internal iliac artery. *D*, Correlation of cinedensitometric measurements and microsphere analysis. The frame count score correlated significantly with blood flow assessment by fluorescent microspheres (normalized to the nonischemic limb).

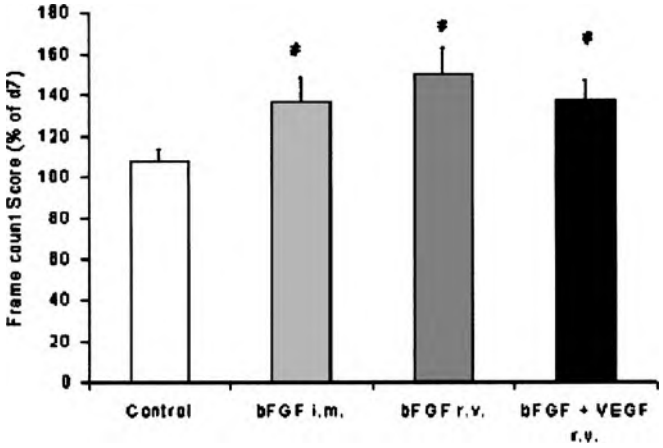

FIG. 5. Ratio of day 35/day 7 frame count score (cf. Materials and Methods) displays a similar increase in blood flow after i.m. (intramuscular), r.v. (retrograde intravenous), and bFGF + VEGF r.v. treatment. $n = 6$, $^{\#}p < .05$ versus saline-retroinfused controls.

As depicted in Figure 5, frame count analysis indicated an increase in blood flow velocity at day 35 in bFGF-treated animals compared to day 7, which did not occur in untreated controls. The increase in frame count score was similar in intramuscularly treated animals. Of note, retroinfusion of bFGF and additional VEGF did not induce a larger increase in frame count score.

DISCUSSION

In the present study, we were able to induce therapeutic angiogenesis as well as arteriogenesis by pressure-controlled retroinfusion of growth factors into a rabbit ischemic hindlimb. Capillary density and collateral growth were increased 28 days after treatment with bFGF, inducing improved collateral blood flow velocity in the ischemic limb. The results did not differ from those obtained after intramuscular injection of bFGF. Importantly, combined retroinfusion of bFGF and low-dose VEGF, which did not induce serious side effects with respect to hypotension or angioma formation, did not further increase blood flow in the hindlimbs compared with bFGF alone.

The application method of growth factors is a crucial factor for the safety and efficacy of therapeutic angiogenesis/arteriogenesis induction in ischemic tissue. For the ischemic heart, mixed results have been obtained following intra-arterial application. For example, intracoronary application of VEGF did not improve myocardial function in one study (Sato et al. 2001). In contrast, intracoronary bFGF induced collateralization and improved myocardial function in a standardized pig model, as opposed to intravenous application (Sato et al. 2000). Reviewing preclinical animal studies, intramuscular application of the proangiogenic agent was suggested as a preferential approach (Losordo et al. 1999). To date, small studies suggest partial benefit of the intramuscular or intra-arterial application of proangiogenic factors, which, however, await conformation in larger studies (for review, see Khan et al. 2003).

Parallel to the ischemic heart, peripheral artery disease has been addressed by growth factor protein or cDNA application, with promising results in experimental (Vajanto et al. 2002; Gowdak et al. 2000) and small patient (Kipshidze et al. 2000; Rajagopalan et al. 2001; Lederman et al. 2002; Lazarous et al. 2000) studies. In the present study, we addressed two possible issues concerning therapeutic efficacy of angiogenesis induction in man: first, we adressed the application route, and second the potential advantage of combination of two growth factors, bFGF and VEGF, as compared to bFGF application alone.

First, intramuscular growth factor application, which is widely used experimentally, but may pose a practical problem in patients, was compared to retrograde intravenous application (retroinfusion). The latter was found to augment retention of a radioactive tracer (^{99m}Tc-nanocolloids) in the ischemic leg severalfold above the level achieved by intra-arterial application at the end of application, an effect lasting for at least 30 min longer (Fig. 1). Moreover, although intramuscular application might best prevent early washout of therapeutic proteins, retroinfusion of bFGF displayed an equipotent improvement of perfusion along with capillary formation and collateral growth (Figs. 2, 3, and 5). Still, retroinfusion of bFGF did not surpass intramuscular bFGF application in terms of therapeutic neovascularization. This comparability, however, might be challenged by, for example, adenovirus-based gene application, which, at least in the myocardial muscle, was more effective and yielded a more homogeneous gene expression than intra-arterial (Boekstegers et al. 2000) or intramuscular application (Boekstegers, unpublished data).

Second, therapeutic angiogenesis might benefit from increased growth factor doses or a combination of the growth factors. With respect to dose escalation, however, several limitations apply: high local VEGF gene delivery by different vectors, such as plasmid DNA, adenovirus, and engineered myoblasts, has been shown to induce hemangioma formation in skeletal muscle, myocardium, and skin (Schwarz et al. 2000; Lee et al. 2000; Springer et al. 1998). Overexpression of VEGF leads to excessive vascular permeability and edema, which has recently been implied as the cause of limb loss (Masaki et al. 2002). Neither of these deleterious effects has been described for bFGF. This proangiogenic factor, acting synergistically with (Yoshiji et al. 2002) and in vitro downstream (Mandriota and Pepper 1997) of VEGF stimulation, is also strongly involved in arteriogenesis initiation (Heidkamp et al. 2002). Because the latter is critical to provide blood flow to the newly formed capillaries, but may not be induced by VEGF-A signaling (Deindl et al. 2001), bFGF application might represent a favorable choice in a clinical setting. In order to investigate whether differing bioactivities of bFGF and VEGF-A would require a coapplication of both factors, we combined bFGF and VEGF-A application. We chose a VEGF-A dose effectively inducing robust angiogenesis in ischemic dog hindlimbs without systemic hypotension and hemangioma formation (10 μg/kg) (Rakue et al. 1998). After combined bFGF and VEGF retroinfusion, we could not detect a

significant increase in capillary formation, collateral growth, or limb perfusion compared to bFGF monotherapy (Figs. 2, 3, and 5), indicating that perfusion of an ischemic hindlimb in vivo is not benefitting from synergism of bFGF and low-dose VEGF-A. Of course, higher doses of VEGF-A act synergistically with bFGF with respect to protein synthesis (Mason et al. 2002), cell migration (Couper et al. 1997), and angiogenesis induction (Asahara et al. 1995). At these concentrations, however, permeability changes, edema formation, and hypotension are at risk again, as demonstrated in rabbits receiving adenovirus transfer of VEGF (Vajanto et al. 2002).

In summary, in the present study, we have established a delivery approach for proangiogenic and proarteriogenic growth factor proteins, which allows for comparable efficacy as intramuscular application but avoiding high local growth factor concentrations. bFGF retroinfusion improved perfusion of an ischemic rabbit hindlimb to a significant extent, which was not further enhanced by additional low-dose VEGF coapplication. These results may translate into a feasible and convenient application mode in patients.

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