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Embryonic chick muscle produces an FGF-like activity

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Abstract. Normal and pathological formation of blood vessels is of considerable interest both in terms of basic scientific processes and clinical applications. Angiogenic events in the adult are likely to represent persistence of developmental mechanisms, and embryos are therefore a suitable experimental model for these processes. Among embryonic tissues, muscle is particularly appropriate for investigation, since it is highly vascularised from early stages. There are a number of competing explanations of how this process is controlled. Bioassays offer advantages over conventional molecular localisation techniques, in that they reveal the presence of active processed forms of the molecules under study, rather than non-processed forms, or non-translated messages. Using these techniques, we report here that embryonic chick muscle, taken from the stages at which blood vessels are forming, produces an angiogenic activity on the chick chorioallantoic membrane (CAM), and transforms NR6 cells in soft agar. Basic fibroblast growth factor (bFGF) is shown to be angiogenic on the CAM in the same way, and also transforms NR6 cells (NR6 cells lack functional epidermal growth factor/transforming growth factor-a receptors, and are believed to respond only to bFGF in this way). Anti-bFGF removes the transforming activity of the embryonic muscle. We conclude that this represents evidence that embryonic chick muscle is producing an FGF-like molecule which is capable of acting as an angiogenic agent at the appropriate times in development.
Key words. Vasculogenesis; angiogenesis; muscle; embryonic; metabolites; fibroblast growth factor (FGF); chorioallantoic membrane (CAM).

Abbreviations. CAM = chorioallantoic membrane; FGF = fibroblast growth factor; aFGF = acidic fibroblast growth factor; bFGF = basic fibroblast growth factor; EGF = epidermal growth factor; TGF = transforming growth factor; VEGF = vascular endothelial growth factor; MEM = minimum essential medium; PBS = phosphate buffered saline; V.D.I. = vascular density index.

Mechanisms of angiogenesis and vasculogenesis are of considerable scientific and clinical importance, and it is likely that valuable clues to the underlying mechanisms can be found by studying the development of blood vessels in the embryo. During development, different tissues are vascularised to varying degrees. Cartilage becomes avascular[1], whereas skeletal muscle has a plentiful blood supply from a relatively early stage of development. This pattern is not predetermined; the tissues themselves influence the growth of capillaries. In embryonic muscle various candidates have been proposed as angiogenic agents, including growth factors, for example basic fibroblast growth factor, bFGF[2], and vascular endothelial growth factor[3], and by-products of muscle metabolism, for example nicotinamide derivatives[4] and adenosine[5].

bFGF has been implicated in angiogenesis[6], inflammation[7] and carcinogenesis[8], among other things. Its role in embryonic development is especially important[9]. aFGF and bFGF are angiogenic factors in avian and mammalian systems in the 10–100 ng range[10–12], showing dose-dependent kinetics[6]. When anti-bFGF antibodies are added to these assays, angiogenesis is inhibited[7]. The secreted peptide vascular endothelial growth factor (VEGF) is also mitogenic for vascular endothelial cells and angiogenic in bioassays[13].

Muscle actively contracts from very early in development[14]. This contraction process will then generate a number of metabolic by-products, and it would be developmentally elegant if such products were themselves angiogenic agents, inducing the growth of blood vessels towards regions of high activity. Such factors (for example nicotinamide derivatives and adenosine) have been reported to induce angiogenesis in in vivo assays[4,5], and it has therefore been hypothesised that this is the major mechanism underlying muscle vascularisation. However, in vivo assays are often difficult to interpret. Factors which cause inflammation, for instance, may induce vascularisation secondarily to this event, rather than as a primary angiogenic agent.

It therefore appears of interest to investigate the production of FGF-like activities by chick embryonic skeletal muscle during the time at which angiogenesis is proceeding. Expression of FGF genes has been observed in mouse embryonic skeletal muscle using RNA in-situ hybridisation[15]. However the approach we have taken employs assays of bioactivity, rather than molec-

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ular probes. This is because it is the presence of the processed active molecule in the extra-cellular environment that is of most interest, and these aspects may not be well revealed by standard techniques[16]. The chick CAM assay was used as a positive control to test the angiogenic activity of bFGF, and as a test of the angiogenic activity of muscle grafts. But as indicated above, there are many confounding factors in such assay systems. An in vitro cellular assay using NR6 cells was also employed. NR6 cells are derived from NRK cells and lack functional EGF receptors[17]. They thus do not transform in anchorage-independence soft agar assays in the presence of EGF, TGFα or TGFβ. However, they do show transformation in response to the presence of bFGF, but not to other single growth factors[18]. NR6 cells are therefore a useful reporter system for the presence of FGF-like molecules.

Materials and methods

In vivo assay

Eggs of the domestic hen, *Gallus gallus* (Muirfield Hatcheries, Kinross, Fife), were incubated blunt end up at 38 °C, and 100% relative humidity. Host eggs were incubated for 7 days and opened via the blunt end. The shell membrane was peeled back to reveal the CAM. Donor eggs were incubated for 4 to 10 days, opened as above, and the embryos removed to 30-mm sterile plastic petri dishes half-filled with α-Eagles' medium (see below). Embryos were staged according to Hamburger and Hamilton[19], decapitated and the hind limbs removed to a fresh dish. The skin was carefully teased away, and cubes of thigh muscle of side 200 µm were cut with electrolytically sharpened tungsten needles. This accuracy was achieved by the use of a photographically reduced 'chess-board' grid of 100-µm squares placed underneath the petri dish. Grafts were then transferred to the host CAM using a fine wire loop, the window sealed with sellotape and the egg returned to the incubator for a further 4 days. Control eggs were handled in exactly the same way, but no graft was made.

Preparation of pellets. Fifty milligram quantities of Elvax 40 (ethylene vinyl acetate) copolymer were placed into 10-ml glass centrifuge tubes, capped with double thickness foil and autoclaved at 6.9×10^4 Pa prior to being dried in a warm air cabinet. Methylene chloride solution (AnalaR) was added to the Elvax 40 at a ratio of 9 ml of methylene chloride per mg of Elvax 40, and this mixture was then allowed to stand until the Elvax 40 had dissolved. Bovine bFGF (Sigma) was added to the mixture to achieve a dose of 10 ng per 10 ml aliquot and mixed thoroughly with a vortex mixer. Then 10-ml aliquots were dispensed onto sterile glass slides to form pellets, and allowed to polymerise at 20 °C for 24–48 h. The slides were placed in a vacuum dessicator at 4 °C for 24–36 h to remove any residual methylene chloride from the pellets and then stored at −20 °C. Sterile precautions were observed throughout. bFGF containing pellets or control pellets made without bFGF were added to the host eggs in exactly the same way as the muscle grafts.

Analysis of CAM grafts. After 4 days, the CAM was fixed in situ with 1 ml of formol saline for 2 min and then removed to a petri dish of phosphate buffered saline (PBS) for quantification of the angiogenic response by the method of Strick et al.[20]. A transparent disc of photographic film with numbered concentric circles of radii of 3.75 mm, 4.69 mm, 5.63 mm and 7.50 mm was placed over the CAM with the centre of the disc over the graft or pellet. The number of blood vessels crossing the circles was counted 3 times, and the average number of intersections for each ring was taken. The vascular density index (V.D.I.) was calculated by dividing the sum of the average number of intersections for the 4 rings by the sum of the circumferences of the 4 rings to give a single reading of vascular density for each CAM, expressed as intersections/mm. Muscle graft experiments were carried out on 9 separate occasions, and the bFGF Elvax 40 pellet experiments were carried out on 6 occasions.

In vitro assay

NR6 cells (a gift from Dr John Heath, then of the University of Oxford) were passaged twice weekly in α-modified Eagles MEM containing 10% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin. Aliquots of 4×10^4 cells were added to 10 ml double-strength medium ($2 \times \alpha$-modified Eagles MEM containing 20% fetal calf serum, 4 mM L-glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin) warmed to 40 °C and mixed by inversion with 0.6% agar which had been boiled for 15 min and then transferred to a 40 °C water bath for 30 min. This double-strength medium also contained 40 mM HEPES buffer. Bovine bFGF (Sigma) and polyclonal rabbit anti-bFGF (Sigma) were added to the double-strength medium prior to mixing with the agar, to give final dilutions as indicated in the section 'Results' and figure legends. Polyclonal anti-bFGF, raised in rabbit[21], was obtained from Sigma. Control solutions were made up to the same volume with medium.

Cell/agar mixture (0.1 ml) was pipetted into each of the 60 central wells of a 96-well flat-bottomed microwell plate, giving approximately 200 cells per well. The peripheral wells of the plate were filled with sterile PBS, and the lidded plates were incubated in a humidified box at 37 °C in 5% CO_2.

Muscle grafts were placed on the top surface of the agar using wire loops. After 7 days, the wells were fixed with a drop of formol saline and examined with an inverted

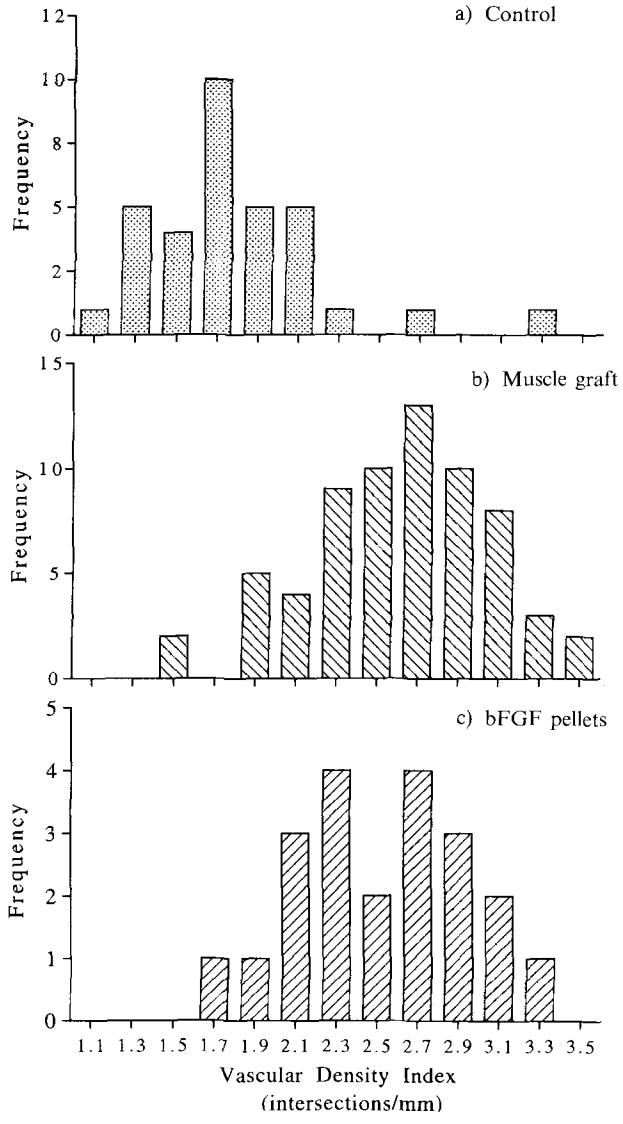

Figure 1. Comparison of angiogenic responses on the chorioallantoic membrane (CAM) assay between controls, embryonic chick muscle grafts and bFGF pellets. The vascular density index (V.D.I.) was calculated by placing a transparent disc with numbered concentric circles over the excised CAM and counting the number of blood vessels crossing 4 of these circles (see text). Each point on the V.D.I. scale shows the mid-point in the range of values from −0.1 to +0.099. (*a*) No significant difference was observed between ungrafted eggs and eggs in which control Elvax 40 pellets lacking bFGF had been placed on the CAM. These control categories are therefore grouped together. (*b*) V.D.I. of eggs which had received grafts to the CAM of muscle cubes of side 200 μm from chick donors of between 4 and 10 days of incubation. (*c*) V.D.I. of eggs which had received grafts of Elvax 40 pellets containing 10 ng bFGF to the CAM. No significant difference was observed in angiogenic response between muscle grafts and bFGF Elvax pellets. bFGF Elvax pellets and muscle grafts were significantly different from controls ($p < 0.0001$).

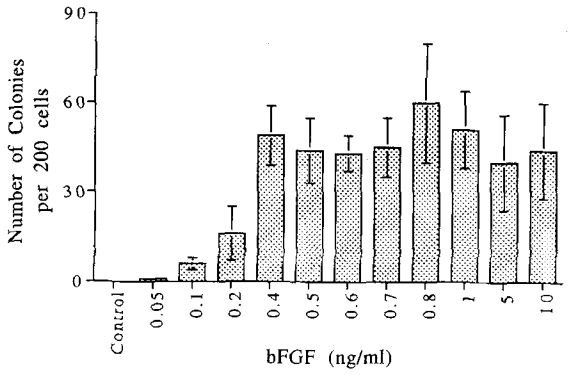

Figure 2. Dose-dependent transformation of the NR6 cell line by bFGF. The transforming ability of bFGF appears to plateau at 0.4 ng/ml. The transformation was measured as the ability to form colonies of 4 or more cells. The number of these colonies was counted in each well, which at the start of the assay contained on average 200 cells. Error bars represent standard deviations.

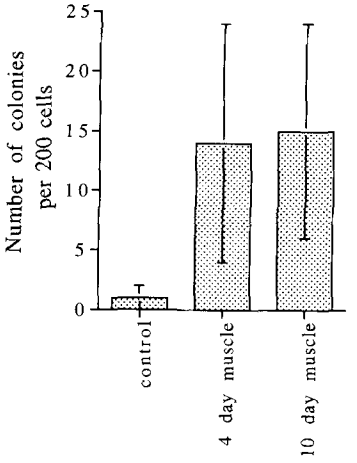

Figure 3. Transformation of the NR6 cell line by embryonic chick muscle grafts at 4 and 10 days of incubation, compared with controls with no grafts. By t-test there is a significant difference between wells containing muscle and control wells ($p < 0.005$). No significant difference was found between 4- and 10-day grafts. Error bars represent standard deviations.

microscope. Clusters of 4 or more cells were scored as a colony. Five internal replicates were carried out for each assay category, and at least two full replicates were carried out on different days.

Statistical analysis

Assay results were tested for normality by n-scores. Results within an assay set were first analysed by two-way analysis of variance. If the assay categories were found not to belong to the same population ($p < 0.05$), sets of results with adjacent means were compared by Student's t-test.

Results

In vivo assays

Results in all in vivo assays were normally distributed. No statistically significant difference was observed between the CAMs of mock operated eggs and eggs which had received control pellet grafts without bFGF (fig. 1a). These groups were therefore pooled for further

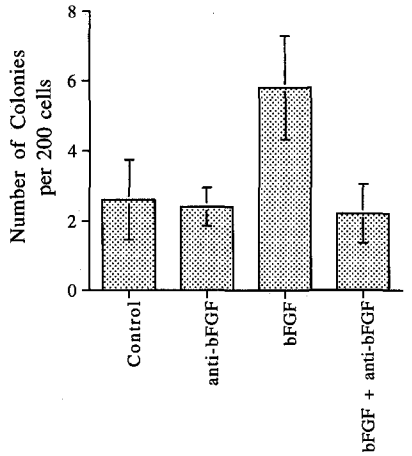

Figure 4. Removal of the ability of bFGF to transform NR6 cells by addition of polyclonal anti-bFGF to the assay. bFGF was present at a final dilution of 5 ng/ml. The antibody was used at a final dilution of 1:400. Error bars represent standard deviations. Results do not all belong to the same population as revealed by analysis of variance: wells containing bFGF are significantly different from the next nearest category by t-test ($p < 0.002$).

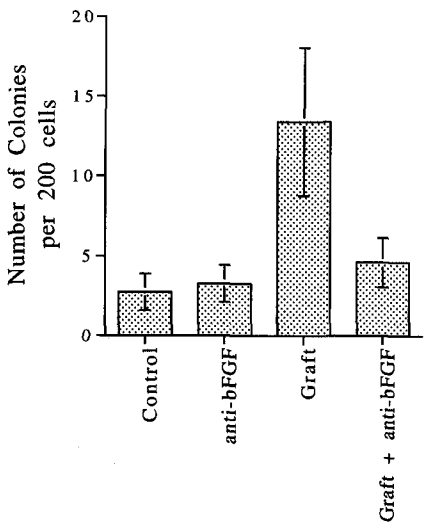

Figure 5. Removal of the transforming ability of 8-day embryonic chick muscle grafts by addition of polyclonal antibody to bFGF to the assay. The antibody was used at a final dilution of 1:800. Results do not all belong to the same population as revealed by analysis of variance: wells containing bFGF are significantly different from the next nearest category by t-test ($p < 0.001$). Error bars represent standard deviations.

analyses. CAMs which had received grafts of muscle cubes or pellets containing 10 ng bFGF showed a positive angiogenic response by comparison to the control group (fig. 1b and c) (significant by t-test at $p < 0.0001$).

In vitro assays

Both bFGF (fig. 2) and muscle explants (fig. 3) induced colony formation by the NR6 cells in soft agar. bFGF had a dose-response effect over the initial part of the range tested (fig. 2), and this then reached a plateau, possibly due to down regulation of receptors.

Addition of anti-bFGF at 1:400 to cultures containing 5 ng bFGF removed the colony-stimulating effect (fig. 4). Cultures containing 5 ng bFGF and anti-bFGF at this titre did not differ significantly from the controls. Anti-bFGF at 1:800 was ineffective at inhibiting growth at comparable levels (results not shown), showing that the ratio of bFGF to anti-bFGF is important[16]. Addition of 1:800 anti-bFGF to cultures containing muscle explants removed the colony-stimulating effect (fig. 5: muscle wells different from next nearest category by t-test at $p < 0.001$). Cultures containing muscle and anti-bFGF together did not differ significantly from the controls.

Discussion

The evidence presented in this paper indicates that both embryonic chick muscle and bFGF are angiogenic on the CAM. Both also produce an activity which stimulates anchorage-independent growth in NR6 cells, which are known to respond in this way to FGF. Extensive computer-based literature searching using Medline and Life Sciences Index failed to reveal another growth factor with this intrinsic ability. The colony-stimulating activity can be entirely abolished from cultures containing bFGF or muscle explants by co-addition of polyclonal bFGF antibody.

A likely conclusion of these observations is that an FGF-like molecule is being produced by embryonic chick muscle at developmental stages corresponding to those at which vascularisation commences. This may play a role in angiogenesis.

Since the muscle cubes were isolated from intact muscle by cutting, it is possible that FGF is released from damaged cells. However, there is at least some evidence that FGF may have an alternative export pathway[22], and it may be secreted by the embryonic muscle. At these early stages, it is not possible to dissect muscle tissue free of other tissues without at least some cutting of the muscle itself.

FGF and an FGF receptor have previously been identified in embryonic chick muscle[15,23,24]. However, the presence of a molecule as detected by molecular localisation techniques is not evidence that the molecule is being produced in an active form, since there may be extensive post-transcriptional and/or post-translational processing required for it to fulfil its biological role.

A relationship has been observed between FGF levels and angiogenesis in a number of embryonic tissues, including brain[25], pituitary[26], heart[27] and CAM[28]. Our observations are consistent with and extend these findings, but they cannot demonstrate that muscle becomes vascularised solely as a consequence of producing an

FGF-like molecule. Developmental mechanisms often feature a degree of apparent redundancy, and it may well be that, for instance, metabolites produced by active muscle also have a role to play in muscle angiogenesis. However, the muscle grafts and explants that we employ are not contracting, since they have been isolated from such innervation as they possessed in the embryo. It therefore seems equally likely that production of metabolites is not the sole mechanism of angiogenesis.

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