

ALTERATIONS OF BLOOD VESSEL DEVELOPMENT BY ENDOTHELIAL CELLS OVEREXPRESSING FIBROBLAST GROWTH FACTOR-2

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SUMMARY

A close relationship exists between angiogenesis and the formation of vascular lesions. The development of the vascular system in the chick embryo chorioallantoic membrane (CAM) may thus represent a model to study the effects of the deregulation of endothelial cell behaviour. Alterations of the developing vascular tree of the CAM were observed after exposure to murine aortic endothelial (MAE) cells overexpressing human fibroblast growth factor-2 (FGF2) cDNA (pZipFGF2 MAE cells), or to their conditioned medium (CM). pZipFGF2 MAE cells injected into the allantoic sac or applied on to the CAM of day 8–9 chick embryos induce neovascularization and the appearance of haemangioma-like lesions. This activity was not prevented by anti-FGF2 antibodies. The CM from pZipFGF2 MAE cells was also active when adsorbed into a gelatin sponge and applied on to the CAM, both in the absence and in the presence of anti-FGF2 antibodies. No effects on vessel development were exerted by parental MAE cells, FGF2-transfected NIH 3T3 fibroblasts, or their conditioned media. *In vitro*, pZipFGF2 MAE cell CM caused parental MAE cells to invade fibrin gels and to undergo morphogenesis on Matrigel. This activity was not mimicked by recombinant FGF2 nor affected by anti-FGF2 antibodies, and depended on a $M_r \sim 45\,000$ heat-labile heparin-binding factor. Size exclusion chromatography of pZipFGF2 MAE cell CM demonstrated that the *in vitro* activity co-purified with an *in vivo* angiogenic capacity. Thus, FGF2 overexpression in mouse endothelial cells induces the production of an angiogenic activity distinct from FGF2, which may contribute to the genesis of angioproliferative lesions. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS—angiogenesis; haemangioma; fibroblast growth factor; development; chick embryo; chorioallantoic membrane

INTRODUCTION

Angiogenesis is characterized by increased proliferation of microvessel endothelial cells, the production and/or activation of matrix degradative enzymes, migration in the subendothelial matrix, and differentiation into functional new capillaries.¹ Endothelial cell turnover is normally very slow in the adult, but physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing. In contrast, the local, uncontrolled release of angiogenic growth factors and/or alterations of the production of natural angiogenic inhibitors, with a consequent alteration of the angiogenic balance,² are thought to be responsible for the uncontrolled endo-

thelial cell proliferation that takes place during tumour neovascularization and in several angioproliferative diseases, including diabetic retinopathy, arthritis, Kaposi's sarcoma, and vascular tumours.^{3,4}

Fibroblast growth factor-2 (FGF2) belongs to the family of the heparin-binding growth factors.⁵ The single copy human FGF2 gene encodes multiple FGF2 isoforms, with M_r s ranging from 18 000 to 24 000. Both low- and high-molecular-weight FGF2 isoforms exert angiogenic activity *in vitro* and induce a pro-angiogenic phenotype in cultured endothelial cells.⁶ FGF2 may exert its effects on endothelial cells via a paracrine mode consequent on its release by tumour and stromal cells and/or by mobilization from proteoglycans of the extracellular matrix.⁷ FGF2 may also play an autocrine role in endothelial cells, as suggested by *in vitro* and *in vivo* experimental evidence (see ref. 8 and refs cited therein). Accordingly, FGF2 has been implicated in the pathogenesis of lesions of endothelial cell origin, including Kaposi's sarcoma (KS)^{9,10} and haemangioma.¹¹

To assess the biological consequences of endothelial cell activation by endogenous FGF2, we originated a stable mouse aortic endothelial cell line (pZipFGF2 MAE cells) transfected with a retroviral expression vector harbouring a human FGF2 cDNA.⁸ FGF2 transfectants show an invasive and morphogenetic behaviour *in vitro*. *In vivo*, they are angiogenic and cause the

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Contract/grant sponsor: Associazione Italiana per la Ricerca sul Cancro.

Contract/grant sponsor: Istituto Superiore di Sanità.

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formation of opportunistic vascular tumours in nude mice, with morphological features resembling KS.^{8,12} These data suggest that FGF2 produced by cells of the endothelial lineage may play important autocrine and paracrine roles in angiogenesis and in the pathogenesis of vascular lesions.

The chick embryo chorioallantoic membrane (CAM) has been utilized widely as a target for angiogenic and anti-angiogenic compounds (see ref. 13 for a review); recombinant FGF2 induces an angiogenic response in the chick CAM.¹⁴ Also, FGF2 mRNA and protein are detectable in the CAM during chicken embryogenesis,^{15,16} and experimental evidence points to a role for endogenous FGF2 in the development of the CAM vasculature.¹⁵

Preliminary macroscopic observations had shown that FGF2-overexpressing pZipFGF2 MAE cells induce alterations of the vascular network of the CAM when injected into the allantoic sac of the chick embryo.⁸ Also, CAM-associated haemangiomas are induced by polyoma virus middle T oncogene (PmT)-transformed mouse endothelial cells (End cells),¹⁷ suggesting that the CAM can be utilized for studying the effects of deregulation of endothelial cell behaviour.

On this basis, we have attempted a characterization of the biological effects and mechanism of action of pZipFGF2 MAE cells on the developing vascular tree of the CAM.

MATERIALS AND METHODS

Cell cultures and preparation of conditioned media

Balb/c mouse aortic endothelial 22106 cells (MAE cells) and human FGF2 cDNA-transfected pZipFGF2 MAE cells^{8,18} were grown in DMEM supplemented with 10 per cent fetal calf serum (FCS) in the absence or in the presence of 500 µg/ml of G418 sulphate (Sigma, St Louis, MO, U.S.A.), respectively. 3T3-HMWbFGF cells¹⁹ were grown in DMEM supplemented with 10 per cent heat-inactivated FCS and 500 µg/ml of G418 sulphate.

Conditioned media were prepared by incubating confluent cell cultures grown in 10 cm dishes with 8 ml of serum-free DMEM for 2–3 days. Media were then collected, clarified by centrifugation, concentrated ten times with M_r 3000 cut-off centrifugal concentrators (Centriplus, Amicon, Beverly, MA, U.S.A.) and stored at -20°C until use. When specified, conditioned media were incubated for 16 h at 4°C with 13 µg/ml neutralizing affinity purified polyclonal anti-FGF2 antibody⁸ or 2.5 µg/ml neutralizing monoclonal anti-FGF2 antibody (Upstate Biotechnology Incorporated, Lake Placid, NY, U.S.A.) before being tested for biological activity. These concentrations of antibody are sufficient to inhibit the mitogenic activity exerted by 10–30 ng/ml FGF2 on cultured endothelial cells (M. Belleri, unpublished observations). In some experiments, pZipFGF2 MAE cell CM was adsorbed on to heparin-Sepharose beads, or incubated for 5 min at 65°C before assay, or concentrated with M_r 30 000 cut-off centrifugal concentrators.

Cell injection into the chick embryo allantoic sac

Fertilized chicken eggs (25 eggs per group) were incubated under routine conditions and a square window was opened in the egg shell at the third day of incubation, after removal of 2–3 ml of albumen to detach the shell from the developing CAM. The window was sealed with a glass of the same size and the eggs were returned to the incubator. Then 200 µl of a cell suspension containing 6×10^6 cells per ml of PBS were injected twice into the allantoic sac at day 8 and day 9 of incubation. At day 12, CAMs were injected with India ink through a large chorioallantoic vein. Specimens were then fixed in Serra's fluid, sectioned, dehydrated in serial alcohols, made transparent in methyl benzoate, and photographed. Morphometric measurements of the vasculature were performed on photographic reconstructions of the CAM at $80 \times$ final magnification by computerized image analysis.²⁰ Data were expressed as a percentage of a 5.0 mm² area of the CAM occupied by the vascular component.

Implantation of gelatin sponges on to the CAM and quantitation of the angiogenic response

Gelatin sponges (Gelfoam, Upjohn Company, Kalamazoo, U.S.A.) were cut to a size of 1 mm³ and placed on top of the CAM at day 8 under sterile conditions.¹⁴ The sponges were then adsorbed with 3 µl of cell suspension (18 000 cells per sponge) or with 5 µl of concentrated CM. Sponges containing vehicle alone were used as negative controls. CAMs were examined daily, photographed *in ovo*, and processed for light microscopy at day 12. Briefly, the embryos and their membranes were fixed *in ovo* in Bouin's fluid. The sponges and the underlying and immediately adjacent portions of CAM were removed and paraffin-embedded. Eight-micrometer serial sections were cut in a plane parallel to the surface of the CAM and stained with a 0.5 per cent aqueous solution of toluidine blue.

The angiogenic response was assessed by a planimetric method of 'point counting'.¹⁴ Briefly, every third section within 30 serial sections from an individual specimen was analysed by a 144-point mesh inserted in the eyepiece of a photomicroscope. Six randomly chosen microscopic fields were evaluated for each section at $250 \times$ magnification. The total number of intersection points occupied by vessels cut transversely (diameters ranging from 3 to 10 µm) inside the sponge and at the boundary between the sponge and the surrounding CAM mesenchyme were counted. Mean values \pm SD were determined for each analysis. The vascular density was indicated by the final mean number of occupied intersection points. The statistical significance of the differences between the mean values of the intersection points in the experimental and control CAMs was determined by the Student *t*-test for unpaired data.

Electron microscopy

Some CAM fragments were fixed *in ovo* in 3 per cent phosphate-buffered glutaraldehyde, dehydrated in serial alcohols, post-fixed in 1 per cent phosphate-buffered

OsO₄, and embedded in Epon 812. Ultrathin sections were cut on an LKB V ultramicrotome in a plane perpendicular to the surface of the CAM and were stained with uranyl acetate followed by lead citrate. Finally, the sections were examined under a Zeiss 9A electron microscope.

Preparation of three-dimensional gels

Twenty-four-well plates were coated with 250 µl/well of 10 mg/ml Matrigel (Becton Dickinson, Milan, Italy) at 4°C. After gelling at 37°C, MAE cells were seeded on to Matrigel layers at 150 000 cells per well.⁸ Culture medium, containing different dilutions of pZipFGF2 MAE cell CM, was renewed every 48 h.

Fibrinogen (2.5 mg/ml) was dissolved in calcium-free medium containing different dilutions of pZipFGF2 MAE cell CM. Then MAE cell aggregates, prepared as previously described,⁸ were resuspended in the fibrinogen solution and clotting was started by addition of thrombin (250 mU/ml). The mixture was transferred into 24-well plates and allowed to gel at 37°C. Trasylol (200 kIU/ml) was added to the gel and to the culture medium to prevent dissolution of the substrate. Cultures were maintained for 2–3 days in DMEM containing the same dilution of CM present within the gel.

Gel filtration and heparin-Sepharose affinity chromatography

100 ml of pZipFGF2 MAE cell CM was concentrated 50 times on to a *M_r* 3000 cut-off centrifugal concentrator. Then 500 µl of the sample (approximately 3.0 mg of protein) was loaded on to a size-exclusion fast protein liquid chromatography (FPLC) Superose-12 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS or in 2.0 M NaCl in PBS and eluted with the same buffer at a flow rate of 1.0 ml/min. One-millilitre fractions were collected, dialysed against fresh medium, and assayed for the capacity to induce MAE cell aggregates to invade fibrin gel as described above. Cultures were maintained for 2–3 days and endothelial cell sprouts were scored by two investigators (M.B. and M.P.) without knowledge of the samples tested. Cultures were graded on an arbitrary scale of 0 to 4+, with 0 representing no sprout-inducing activity and 4+ representing the strongest activity. Molecular size standards were chromatographed under the same experimental conditions.

In a second set of experiments, 10 ml of pZipFGF2 MAE cell CM was loaded on to a 0.5 ml heparin-Sepharose column equilibrated in PBS. The column was then washed with 10 ml of PBS and eluted step-wise with increasing concentrations of NaCl in PBS (1.5 ml/step). Fractions were collected, dialysed against fresh medium, and assayed for their sprout-inducing capacity as described above.

RESULTS

pZipFGF2 MAE cells induce angiogenesis and haemangioma formation in chick embryo CAM

When 1.2×10^6 pZipFGF2 MAE cells were injected twice into the allantoic sac of chick embryos at days 8

and 9, significant modifications of the developing vasculature of the CAM were observed: blood vessels with an irregular course and frequent branching (average vascular density equal to 9.74 per cent of the CAM surface) were present at day 12 (Fig. 1A). In contrast, blood vessels ran straight and interdigitated regularly in the CAM of embryos injected with parental MAE cells or vehicle (average vascular density 7.43 and 7.22 per cent, respectively). Also, intravenous (i.v.) injection of India ink revealed the presence of ink-filled enlarged areas scattered within the blood vessel network of pZipFGF2 MAE cell-treated CAMs (Fig. 1A, arrows). PmT-transformed End cells induce haemorrhaging and haemangiomas at the site of injection.¹⁷ To investigate the nature of the ink-filled areas observed in the CAM of pZipFGF2 MAE cell-treated embryos, tissues were examined at the histological and ultrastructural level. As shown in Figs 1B and 1C, multiple haemangioma-like lesions of various sizes surrounded by numerous capillaries were present in pZipFGF2 MAE cell-treated CAMs. These lesions are characterized by enlarged, blood-filled sacs lined by a thin endothelial cell monolayer. Careful examination of serial sections showed no signs of thrombotic and/or haemorrhagic lesions. These data were confirmed by histological analysis of ink-injected CAMs. Indeed, as shown in Figs 1D and 1E, India ink was evident within the endothelial cell-lined enlarged cavernae and the surrounding small blood vessels, but was undetectable in the stroma. Similar results were obtained with two MAE cell clones that produce levels of FGF2 protein comparable to those expressed by pZipFGF2 MAE cells and that originated from independent FGF2 transfection experiments (data not shown).

pZipFGF2 MAE cells induce opportunistic vascular lesions when injected subcutaneously in nude mice.¹² FGF2-overexpressing cells can be isolated from these lesions after G418 selection *in vitro*. These cells retain several properties of pZipFGF2 MAE cells¹² and they also show the capacity to induce angiogenesis and haemangioma formation when injected into the allantoic sac of the chick embryo (data not shown). No alterations of the developing vasculature of the CAM were observed in embryos treated with parental or mock transfected MAE cells, or in control embryos.

pZipFGF2 MAE cells treated for 3 h with mitomycin C (10 µg/ml) before injection into the allantoic sac, a concentration of the drug sufficient to block their proliferation *in vitro*, were still able to induce a vasoproliferative response and the formation of haemangiomas. In contrast, mild fixation of the cells with glutaraldehyde completely abolished their angiogenic and haemangioma-inducing activity. These data indicate that the injection of live, non-proliferating FGF2-transfected MAE cells is sufficient to induce the observed modifications of the CAM vasculature that are therefore due to alterations of the behaviour of the endothelial cells of the host.¹⁷

To assess whether the capacity to induce these alterations was shared by any murine FGF2-overexpressing cell line, 3T3-HMWbFGF cells, a FGF2-transfected NIH 3T3 cell line that expresses high levels of all

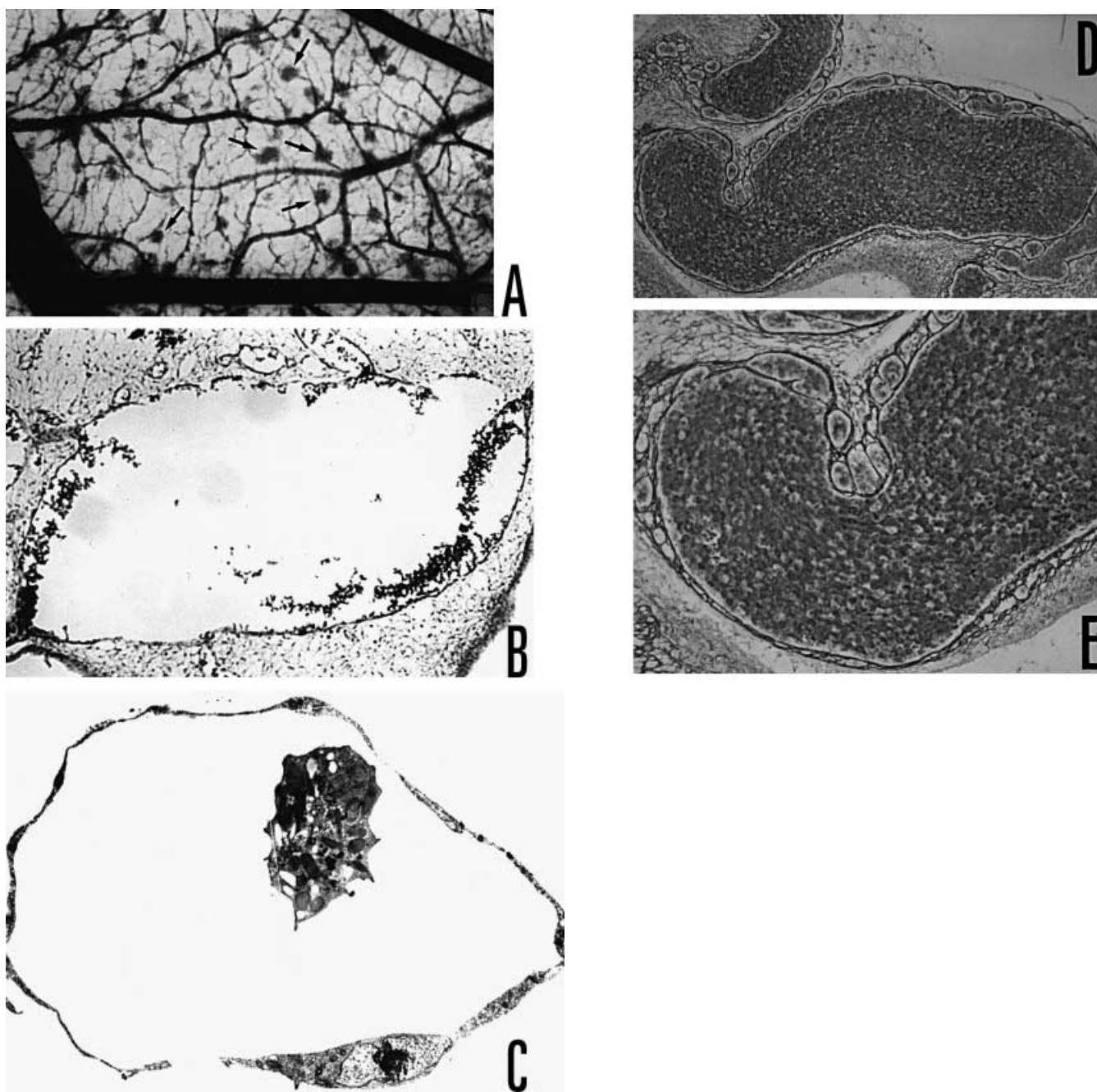


Fig. 1—Alterations of the CAM vasculature following injection of pZipFGF2 MAE cells into the allantoic sac of chick embryo. Cells were inoculated twice at days 8 and 9 and the CAM was studied at day 12. (A) Photographic reconstructions of the CAM vasculature visualized after i.v. injection of India ink. Note the irregular course, branching, the high density of CAM vasculature, and the presence of numerous haemangiomas of various sizes (arrow-heads). Histological analysis (B) and transmission electron microscopy (C) showed isolated haemangiomas filled by blood cells and lined by a very thin endothelium in the mesenchyme of CAM injected with pZipFGF2 MAE cells. (D) Histological analysis of an India ink-injected CAM showing an enormous haemangioma filled by ink that completely occupies the CAM mesenchyme. Numerous newly formed small blood vessels underlying the chorionic epithelium are also recognizable. At higher magnification (E), note the continuity of the endothelium limiting the haemangioma. (A) $\times 140$; (B) $\times 400$; (C) $\times 4500$; (D) $\times 100$; (E) $\times 250$ —all magnification reduced to 60 per cent in printing

FGF2 isoforms,¹⁹ were injected into the allantoic sac under the same experimental conditions. No haemangiomas and no significant modifications of the vascular density were observed in the CAM of these embryos when compared with the CAM of embryos injected with parental 3T3 fibroblasts or control embryos (average vascular density 7.30, 7.10, and 7.22 per cent, respectively).

pZipFGF2 MAE cells secrete approximately 100 pg of FGF2/96 h per 10^6 cells, as evaluated by immunoassay (Quantikine, R&D Systems, Minneapolis, MN, U.S.A.). Thus, they are anticipated to release approximately 250 pg of FGF2 throughout the experimental period, when injected into the allantoic sac according to our experimental protocol. This amount is significantly lower than the minimal amount required to induce an

angiogenic response in the CAM when FGF2 is applied on the top of the membrane (approximately 10–30 ng/sample²¹). Moreover, the route of administration dictates the angiogenic activity of human recombinant FGF2 in the CAM, the growth factor being ineffective when injected into the allantoic sac, even at doses as high as 4 µg per egg.¹⁵ These data suggest that the angiogenic and haemangioma-inducing activity exerted by pZipFGF2 MAE cells injected into the allantoic sac may not depend on the release of FGF2 into the allantoic fluid. To assess this hypothesis, 1.2×10^6 pZipFGF2 MAE cells were injected twice into the allantoic sac together with 400 ng of affinity-purified, neutralizing anti-FGF2 polyclonal antibody. Even though this amount of antibody was sufficient to neutralize the biological activity exerted by 2.5–5.0 ng of FGF2 on cultured endothelial cells (M. Belleri, unpublished observations), it had no effects on the modifications of CAM vasculature induced by pZipFGF2 MAE cells (data not shown).

pZipFGF2 MAE cells release an angiogenic activity distinct from FGF2

To evaluate whether the angiogenic activity of pZipFGF2 MAE cells is due to diffusible factor(s), we assessed the activity of serum-free pZipFGF2 MAE cell CM that was concentrated ten times using a M_r 3000 cut-off centrifugal concentrator. To avoid its dilution into the allantoic fluid, the concentrated CM (5 µl) was adsorbed into a gelatin sponge and applied on the top of the CAM.¹⁴ Live pZipFGF2 MAE cells were delivered on to the CAM under the same experimental conditions as positive controls (1.8×10^4 cells per sponge). Concentrated CM from parental MAE cells and live MAE cells were utilized as negative controls. After 4 days, macroscopic observation of the CAMs showed that the sponges treated with pZipFGF2 MAE cells or their concentrated CM (Fig. 2A) were surrounded by numerous allantoic vessels which developed radially towards the implant in a 'spoked wheel' pattern. Also, scattered haemangiomas were recognizable in close proximity to the sponge (Fig. 2B). In the specimens treated with MAE cells or their CM, no vascular response or haemangiomas were detectable around the sponges.

Histological examination of the sponges treated with pZipFGF2 MAE cells or their CM revealed a collagenous matrix containing numerous small blood vessels among the sponge trabeculae (Fig. 3A). At the boundary between the sponge and the surrounding CAM mesenchyme, numerous host capillaries were recognizable, penetrating in some points into the sponge (Fig. 3B). In contrast, no blood vessels or collagenous matrix was present among the sponge trabeculae in the specimens treated with MAE cells or their CM. Quantitation of the angiogenic response demonstrated a significant increase in blood vessel density both within the gelatin sponge and at the boundary between the sponge and the CAM mesenchyme, for implants treated with pZipFGF2 MAE cells or with their CM (Table I).

In agreement with the results obtained after injection into the allantoic sac, FGF2-transfected and parental

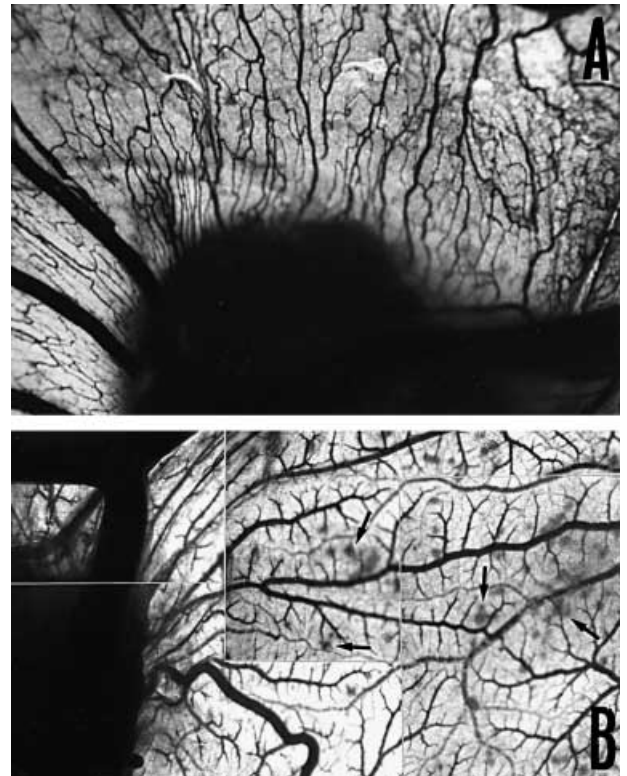


Fig. 2—Alterations of the CAM vasculature after implantation of a gelatin sponge adsorbed with concentrated pZipFGF2 MAE cell CM. The CAM vasculature was visualized after 4 days by i.v. injection of India ink. Note the numerous allantoic vessels growing towards the sponge in a 'spoked wheel' pattern (A) and the numerous haemangiomas in close proximity to the sponge (arrow-heads in B). $\times 80$, reduced to 57 per cent in printing

3T3 cells and their conditioned media did not exert any effect on the CAM vasculature when adsorbed into the sponges (data not shown). Moreover, the angiogenic activity of pZipFGF2 MAE cells was not affected when cells were applied on to the CAM together with 400 ng of neutralizing anti-FGF2 antibody (Table I). Also, anti-FGF2 antibody adsorbed into the sponge had no effects on the modifications of the CAM vasculature induced by the injection of pZipFGF2 MAE cells into the allantoic sac (data not shown). In conclusion, the data indicate that pZipFGF2 MAE cells release an angiogenic activity distinct from FGF2.

Characterization of the angiogenic activity released by pZipFGF2 MAE cells

pZipFGF2 MAE cells release limited amounts of FGF2 (see above). Nevertheless, the above experiments indicated that pZipFGF2 MAE cells may also secrete factor(s) distinct from FGF2 and responsible for their capacity to cause the observed deregulation of endothelial cell behaviour of the CAM.

Since vascular organization requires morphogenesis and endothelial cell invasion of the surrounding matrix, we evaluated the effect of the CM of pZipFGF2 MAE cells on the morphogenic and invasive potential of cultured endothelial cells.⁸ When seeded on Matrigel,

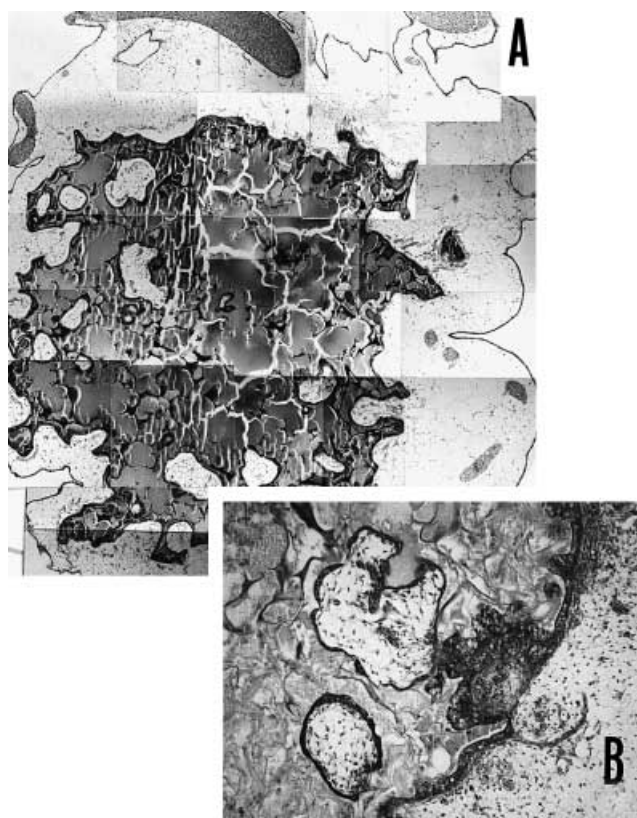


Fig. 3—Photographic reconstruction of a histological section parallel to the CAM surface showing a gelatin sponge adsorbed with a suspension of pZipFGF2 MAE cells. Numerous small blood vessels in a collagenous matrix are present among the sponge trabeculae (A). At higher magnification, numerous vessels at the boundary between the sponge and the CAM mesenchyme penetrate the implant (B). Similar results were obtained when the sponge was adsorbed with 5 μ l of concentrated pZipFGF2 MAE cell CM

parental MAE cells originate a complex network of branching cord-like structures after 6–12 h of culture in the presence of pZipFGF2 MAE cell CM, but not in the presence of fresh medium or medium conditioned by parental MAE cells (Fig. 4). The effect was dose-

dependent and stimulation was maximal when the concentrated CM was diluted 1:10–1:30 with fresh medium. Also, morphogenesis of MAE cells grown in the presence of pZipFGF2 MAE cell CM was not prevented by neutralizing anti-FGF2 antibodies. Finally, MAE cells did not undergo morphogenesis when grown on Matrigel in fresh culture medium supplemented with vehicle, recombinant FGF2, vascular endothelial growth factor₁₆₅ (VEGF₁₆₅), and hepatocyte growth factor (HGF) (Fig. 4). The morphogenic capacity of the pZipFGF2 MAE cell CM was abolished by incubation at 65°C for 5 min before the assay (Fig. 4), or when it was pre-absorbed on to heparin-Sepharose beads (data not shown). pZipFGF2 MAEC CM retained instead full morphogenic activity when concentrated using a M_r 30 000 cut-off centrifugal concentrator.

In a second set of experiments, aggregates of parental MAE cells were grown in fibrin gel containing different dilutions of pZipFGF2 MAE cell CM. Under these experimental conditions, MAE cells invade the gel and form sprouts radiating out from the aggregates after 2 days in culture (Fig. 5). As observed for the morphogenic assay, the effect was dose-dependent and stimulation was maximal when the concentrated CM was diluted 1:10–1:30 with fresh medium. Also, sprout formation was not prevented by addition of either monoclonal (Fig. 5) or polyclonal (not shown) neutralizing anti-FGF2 antibodies within the gel. Conversely, cells did not migrate and invade fibrin when aggregates were embedded in gels containing fresh culture medium supplemented with vehicle or recombinant FGF2, VEGF₁₆₅, or HGF, or with concentrated medium that was conditioned by parental MAE cells (Fig. 5).

To test whether the sprout-inducing activity could be isolated by affinity chromatography on heparin, pZipFGF2 MAE cell CM was applied on to a heparin-Sepharose column. The column was then eluted stepwise with increasing concentrations of NaCl in PBS. Fractions were dialysed against fresh medium and assayed for the capacity to induce parental MAE cell aggregates to invade fibrin gel. The results indicate that

Table I—Angiogenic activity of pZipFGF2 MAE cells and their CM in CAM*

Treatment	Blood vessels at intersection points	
	Inside the sponge	Boundary between sponge and CAM mesenchyme
Vehicle	0	6 ± 1.2
MAE cells	0	5 ± 1.5
MAE cell CM	0	4 ± 1.9
pZipFGF2 MAE cells	26 ± 3.2†	34 ± 6.2†
pZipFGF2 MAE cell CM	30 ± 1.5†	35 ± 5.8†
pZipFGF2 MAE cell CM plus anti-FGF2 antibody (400 ng)	27 ± 2.6†	37 ± 2.5†

*MAE cells, pZipFGF2 MAE cells (both at 1.8×10^4 cells in 3 μ l of PBS), or 10-fold concentrated conditioned media (5 μ l) were absorbed into gelatin sponges implanted onto the CAM at day 8 (ten embryos per group). The angiogenic response was assessed by a planimetric method of 'point counting' at day 12 as described in the Materials and Methods section.

†Significantly different from vehicle ($p < 0.001$).

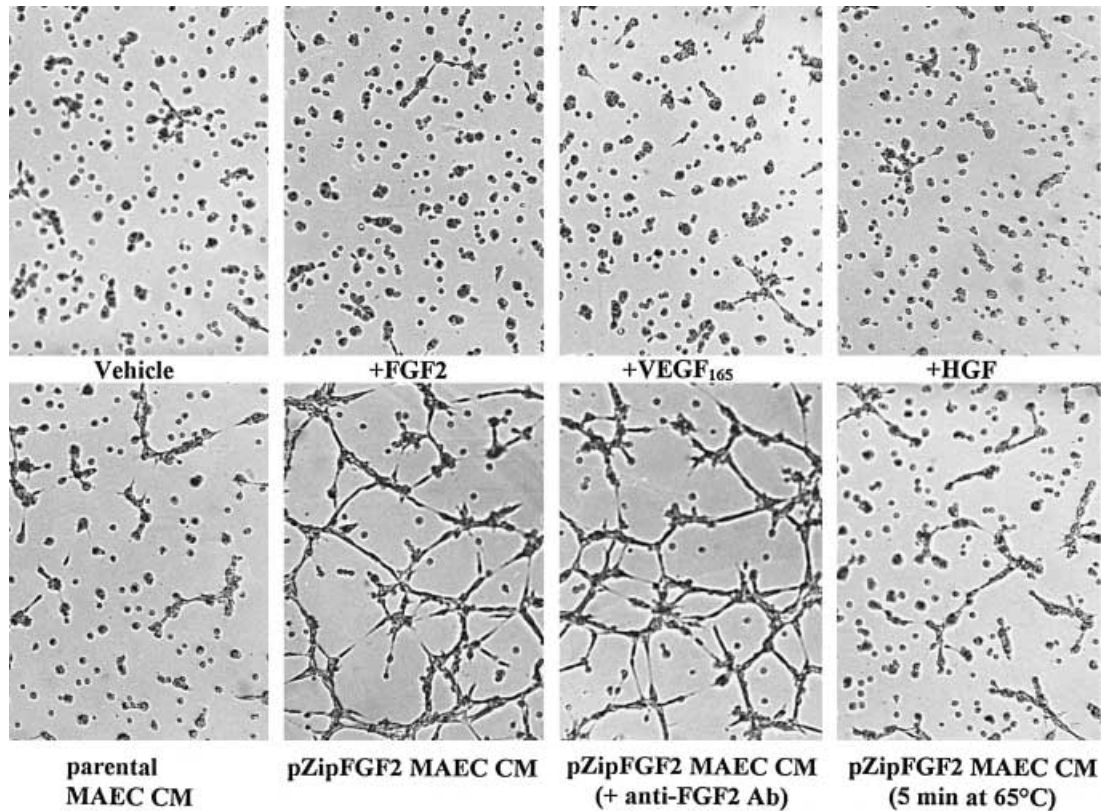


Fig. 4—Morphogenetic activity of pZipFGF2 MAE cell CM. Parental MAE cells were seeded on Matrigel and grown in fresh DMEM supplemented with vehicle, recombinant FGF2 (30 ng/ml), VEGF₁₆₅ (100 ng/ml), HGF (30 ng/ml), or with concentrated medium that was conditioned by parental MAE cells or by pZipFGF2 MAE cells (both at 1:5 final dilution). Some cultures were treated with pZipFGF2 MAE cell CM that was incubated for 16 h at 4°C with neutralizing monoclonal anti-FGF2 antibody (2.5 µg/ml) or neutralized for 5 min at 65°C before the assay. All cell cultures were photographed using an inverted phase contrast photomicroscope 7 h after the beginning of the treatment. × 40, reduced to 80 per cent in printing

all the activity binds to the column from where it is eluted as a single peak at 1.0 M NaCl (Fig. 6A). In a parallel experiment, pZipFGF2 MAE cell CM was concentrated approximately 50 times using a M_r 3000 cut-off centrifugal concentrator, fractionated on to a size-exclusion fast protein liquid chromatography (FPLC) Superose-12 column equilibrated at low ionic strength in PBS, and fractions were tested for sprout-inducing activity. Two peaks of activity with apparent molecular weights equal to ~200 000 and ~45 000 were observed (Fig. 6B). When fractionation of pZipFGF2 MAE cell CM was repeated using the same size-exclusion FPLC column under higher ionic strength conditions (2.0 M NaCl in PBS), all the activity eluted instead as a single peak at M_r ~45 000 (Fig. 6C). This activity binds heparin-Sepharose beads and is not affected by incubation with neutralizing monoclonal anti-FGF2 antibodies. Finally, when the size-exclusion FPLC column fractions were adsorbed into gelatin sponges and tested in the chick embryo CAM assay, the results demonstrated that the angiogenic activity of pZipFGF2 MAE cell CM co-purifies with the *in vitro* endothelial sprout-inducing capacity (data not shown). However, no haemangiomas were observed in the CAMs treated with FPLC column fractions endowed with angiogenic activity.

DISCUSSION

The development of the vascular system of the chick embryo CAM is a complex, highly regulated process that depends on genetic and epigenetic factors expressed by endothelial and non-endothelial cells;¹⁶ it may therefore represent a useful model to study the effects of the deregulation of endothelial cell behaviour. This study shows that FGF2-overexpressing mouse endothelial cells affect this developmental process by inducing an increase in vascular density and the formation of angioproliferative lesions when injected into the allantoic sac or implanted on the top of the CAM. No effects on CAM vessel development were exerted by parental MAE cells. These lesions are due to alterations of the host developmental process that result in increased proliferation of vascular endothelial cells and the loss of their normal morphogenetic programme, thus causing increased neovascularization and the formation of haemangioma-like lesions. These lesions consist of blood-filled sacs of various sizes lined by a thin layer of host endothelial cells, in the absence of thrombosis and haemorrhage. It must be pointed out that the capacity to affect blood vessel development of the CAM is shared by two other independent FGF2-transfected MAE clones and by pZipFGF2 MAE cells after *in vivo*

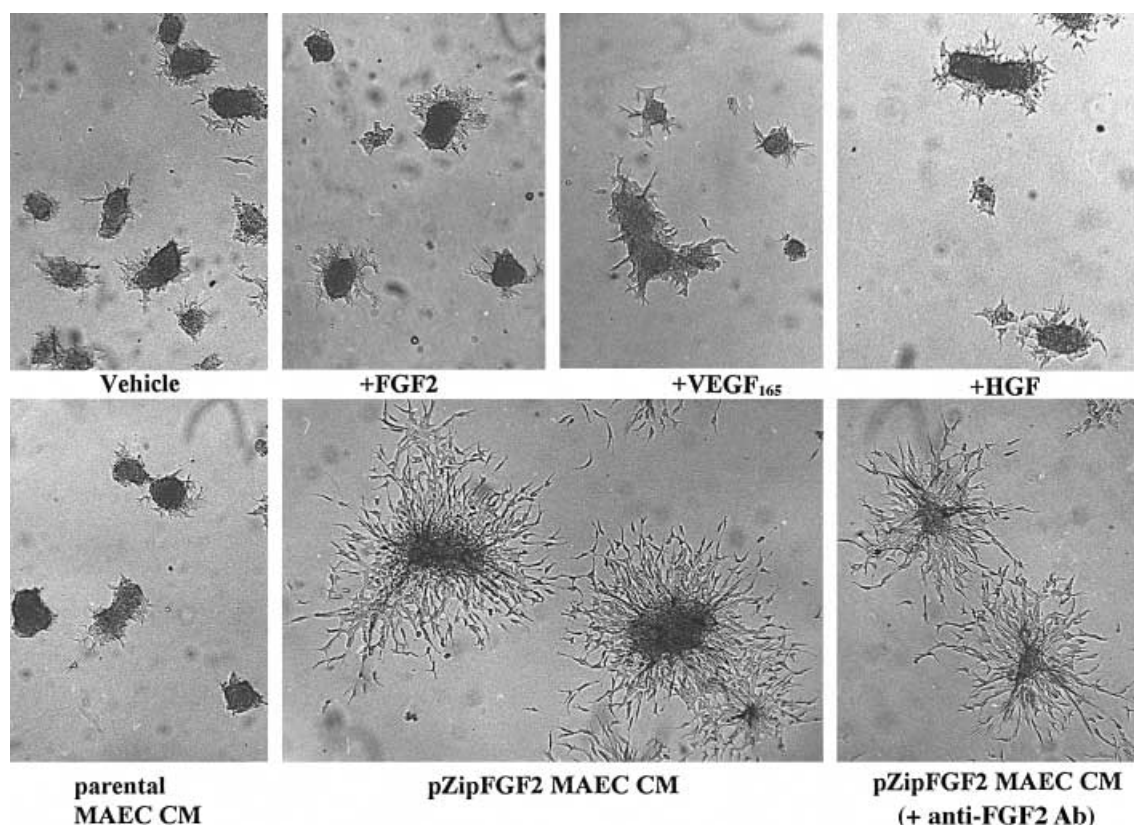


Fig. 5—Endothelial cell sprout formation induced by pZipFGF2 MAE cell CM. Spheroid aggregates of parental MAE cells were grown within fibrin gels in fresh DMEM supplemented with vehicle, recombinant FGF2 (30 ng/ml), VEGF₁₆₅ (100 ng/ml), HGF (30 ng/ml), or with concentrated medium that was conditioned by parental MAE cells or by pZipFGF2 MAE cells (both at 1:5 final dilution). Some cultures were treated with pZipFGF2 MAE cell CM that was incubated for 16 h at 4°C with neutralizing monoclonal anti-FGF2 antibody (2.5 µg/ml). All cell cultures were photographed using an inverted phase contrast photomicroscope 2 days after the beginning of the treatment. × 40, reduced to 80 per cent in printing

passage into nude mice. In contrast, 3T3 fibroblasts overexpressing FGF2 did not cause any alterations of the CAM vasculature under the same experimental conditions.

The effects exerted by pZipFGF2 MAE cells on CAM blood vessels resemble in part those elicited by PmT-transformed mouse End cells.¹⁷ End cells cause the growth of haemangiomas by recruitment of the endothelial cells of the host when injected into the coelomic cavity or into chorioallantoic or yolk sac veins.¹⁷ In this case also, the haemangioma-inducing capacity appeared to be limited to endothelial cells, since PmT-transformed fibroblasts were ineffective. However, pZipFGF2 MAE cells differ from End cells in their angiogenic potential; they and their CM are able to exert a potent angiogenic activity in chick CAM, whereas no increase in CAM vascularization can be induced by extracts of End cells or their CM.¹⁷

Our data indicate that the limited amounts of biologically active FGF2 released by pZipFGF2 MAE cells do not fully account for their angiogenic and haemangioma-inducing capacity. Firstly, recombinant FGF2 induces neovascularization, but does not cause haemangioma formation when applied on the top of the CAM;¹⁵ secondly, injection of recombinant FGF2 into the allantoic sac is ineffective;¹⁵ thirdly, neutralizing anti-FGF2 antibodies do not abolish neovascularization

and haemangioma formation induced by pZipFGF2 MAE cells or by their CM; and fourthly, 3T3 fibroblasts overexpressing FGF2 and their CM are ineffective. Thus, the observed alterations of the CAM vasculature elicited by pZipFGF2 MAE cells appear to be due to factor(s) distinct from FGF2, rather than to a direct action of released FGF2.

In agreement with this hypothesis, the CM of pZipFGF2 MAE cells induces a morphogenetic and invasive response in cultured endothelial cells that could not be mimicked by recombinant FGF2 (nor by VEGF₁₆₅ or HGF) and was not prevented by co-incubation with neutralizing anti-FGF2 antibody. This response is due to a $M_r \sim 45\,000$ heparin-binding, heat-labile factor released by FGF2-transfected MAE cells. Under physiological conditions, this factor forms biologically active, high-molecular-weight complexes that can be dissociated at high ionic strength. This factor may be responsible, at least in part, for the alterations of the CAM vasculature induced by pZipFGF2 MAE cells, as indicated by the observation that it co-purifies with an angiogenic activity when the pZipFGF2 MAE cell CM is fractionated by size-exclusion chromatography. However, its inability to induce haemangioma formation in the CAM indicates that other factors produced by pZipFGF2 MAE cells may be involved in the genesis of these lesions. Also, our data do not rule out the

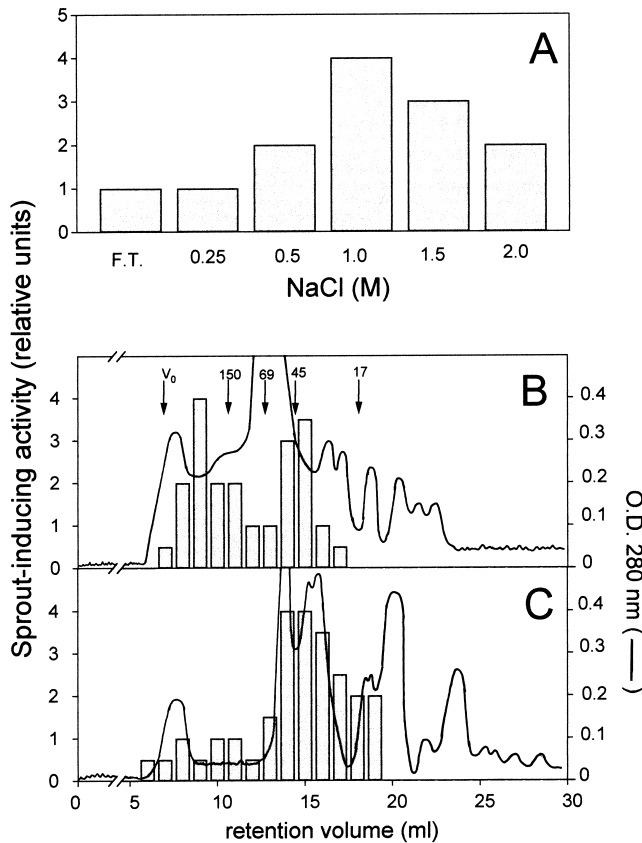


Fig. 6—Gel filtration and heparin-Sepharose affinity chromatography of pZipFGF2 MAE cell CM. (A) 10 ml of pZipFGF2 MAE cell CM was loaded on to a 0.5 ml heparin-Sepharose column equilibrated in PBS. The column was then washed with 10 ml of PBS and eluted step-wise with increasing concentrations of NaCl in PBS (1.5 ml/step). Fractions were dialysed and assayed for the capacity to induce MAE cell aggregates to invade fibrin gel. MAE cell sprouts were scored without knowledge of the samples tested and graded on an arbitrary scale of 0 to 4+, with 0 representing no sprout-inducing activity and 4+ representing the strongest activity. F.T.=flow-through of the column. (B, C) 100 ml of pZipFGF2 MAE cell CM was concentrated 50 times using a M_r 3000 cut-off centrifugal concentrator. Then 500 μ l of the sample was loaded on to an FPLC Superose-12 column equilibrated in PBS (B) or in 2.0 M NaCl in PBS (C) and eluted with the same buffer at 1.0 ml/min. Fractions were collected, dialysed, and assayed for their sprout-inducing capacity. Molecular sizes are in thousands. The results shown are representative of two independent experiments that gave similar elution profiles

possibility that released FGF2 may cooperate with these factor(s) in inducing the observed vascular alterations of the CAM.

pZipFGF2 MAE cells and parental MAE cells do not express VEGF, placenta growth factor or interleukin-6 and have very low levels of angiopoietin-1 and angiopoietin-2 mRNAs, two genes involved in blood vessel maturation and maintenance, angiogenesis, and regression.²² Moreover, the conditioned media of parental MAE cells and of pZipFGF2 MAE cells exert a similar chemotactic activity for human umbilical vein endothelial cells and murine macrophages and contain similar levels of the chemokine MCP-1 α and of latent transforming growth factor- β , whereas both cell types secrete undetectable levels (<1 ng/ml) of thrombospondin-1 (data not shown). Purification to

homogeneity and amino acid sequencing will be required for an unambiguous identification of the molecule responsible for the observed effects exerted *in vitro* and *in vivo* by pZipFGF2 MAE cells and their CM on vascular endothelium.

Experimental evidence implicates FGF2 in angiogenesis and in the formation of vascular lesions. Endogenous FGF2 exerts autocrine effects on endothelial cells^{23–25} and on KS spindle cells of endothelial origin.¹⁰ Accordingly, subcutaneous injection of FGF2 in nude mice causes the appearance of vascular lesions characteristic of early-stage KS.⁹ FGF2 expression has also been demonstrated in endothelial cells during the proliferative phase of human haemangiomas.¹¹ Finally, FGF2-overexpressing endothelial cells acquire an angiogenic phenotype and recruit quiescent endothelium, originating vascular lesions *in vivo* in different animal species^{8,12} (present work). Our data indicate that endogenous FGF2 may act indirectly by inducing the production of an angiogenic factor by endothelial cells, as already shown for the FGF2-mediated expression of VEGF and MCP-1.^{26,27} FGF2 has been hypothesized to exert intracellular functions.^{28,29} The inability of neutralizing anti-FGF2 antibodies to prevent the alterations of the vascular tree of the CAM induced by pZipFGF2 MAE cells *in vivo* suggest that FGF2 may stimulate the production of this angiogenic factor by an intracellular mode of action. In turn, this factor may contribute to the genesis of angioproliferative lesions by autocrine and paracrine mechanisms.

The identification of the molecule responsible for the observed effects exerted *in vitro* and *in vivo* by bZipFGF2 MAE cells on vascular endothelium may help to elucidate the mechanisms of regulation of blood vessel growth and morphogenesis that occur during physiological and pathological conditions. In addition, the chick embryo CAM/FGF2-overexpressing pZipFGF2 MAE cell system may represent a useful model to study the genesis of vascular lesions characterized by intense neovascularization and host endothelial cell recruitment.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Associazione Italiana per la Ricerca sul Cancro to D.R., A.V., and M.P. (Special Project Angiogenesis); Istituto Superiore di Sanità (X AIDS Project, 1997); MURST (Project Inflammation: Biology and Clinics); and Centro per lo Studio del Trattamento dello Scompenso Cardiaco to M.P.

REFERENCES

1. Risau W. Mechanisms of angiogenesis. *Nature* 1997; **386**: 671–674.
2. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996; **86**: 353–364.
3. Enzinger FM, Weiss SW. *Soft Tissue Tumors*. St Louis: Mosby-Year Book, 1995: 579–677.
4. Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med* 1995; **333**: 1757–1763.
5. Basilico C, Moscatelli D. The FGF family of growth factors and oncogenes. *Adv Cancer Res* 1992; **59**: 115–165.
6. Gualandris A, Urbinati C, Rusnati M, Ziche M, Presta M. Interaction of high molecular weight basic fibroblast growth factor (bFGF) with

- endothelium: biological activity and intracellular fate of human recombinant M_r 24,000 bFGF. *J Cell Physiol* 1994; **161**: 149–159.
7. Bikfalvi A, Klein S, Pintucci G, Quarto N, Rifkin DB. Biological roles of fibroblast growth factor-2 (FGF-2). *Endocr Rev* 1997; **18**: 26–45.
 8. Gualandris A, Rusnati M, Belleri M, *et al.* Basic fibroblast growth factor overexpression in endothelial cells: an autocrine mechanism for angiogenesis and angioproliferative diseases. *Cell Growth Differ* 1996; **7**: 147–160.
 9. Ensoli B, Gendelman R, Markham P, *et al.* Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature* 1994; **371**: 674–680.
 10. Ensoli B, Markham P, Kao V, *et al.* Block of AIDS–Kaposi's sarcoma cell growth, angiogenesis, and lesion formation in nude mice by antisense oligonucleotide targeting basic fibroblast growth factor. A novel strategy for the therapy of KS. *J Clin Invest* 1994; **94**: 1736–1746.
 11. Takahashi K, Mulliken JB, Kozakewich HPW, Rogers RA, Folkman J, Ezekowitz RAB. Cellular markers that distinguish the phases of hemangioma during infancy and childhood. *J Clin Invest* 1994; **93**: 2357–2364.
 12. Sola F, Gualandris A, Belleri M, *et al.* Endothelial cells overexpressing basic fibroblast growth factor (FGF2) induce vascular tumors in immunodeficient mice. *Angiogenesis* 1997; **1**: 102–116.
 13. Ribatti D, Vacca A, Roncali L, Dammacco F. The chick embryo chorioallantoic membrane as a model for *in vivo* research on angiogenesis. *Int J Dev Biol* 1996; **40**: 1189–1197.
 14. Ribatti D, Gualandris A, Bastaki M, Vacca A, Roncali L, Presta M. A new model for the study of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane (CAM): the gelatin sponge/CAM assay. *J Vasc Res* 1997; **34**: 455–463.
 15. Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, Presta M. Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Dev Biol* 1995; **170**: 39–49.
 16. Ribatti D, Bertossi M, Nico B, *et al.* Role of the basic fibroblast growth factor in the formation of the capillary plexus in the chick embryo chorioallantoic membrane. An *in situ* hybridization, immunohistochemical and ultrastructural study. *J Submicr Cytol Pathol* 1998; **30**: 127–136.
 17. Williams RL, Risau W, Zerwes HG, Drexler H, Aguzzi A, Wagner EF. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell* 1989; **57**: 1053–1063.
 18. Bastaki M, Nelli EE, Dell'Era P, *et al.* Basic fibroblast growth factor-induced angiogenic phenotype in mouse endothelium. A study of aortic and microvascular endothelial cell lines. *Arterioscler Thromb Vasc Biol* 1997; **17**: 454–464.
 19. Gualandris A, Coltrini D, Bergonzoni L, *et al.* The NH₂-terminal extension of high molecular weight forms of basic fibroblast growth factor (bFGF) is not essential for the binding of bFGF to nuclear chromatin in transfected NIH 3T3 cells. *Growth Factors* 1993; **8**: 49–60.
 20. Ribatti D. A morphometric study of the expansion of the chick area vasculosa in shell-less culture. *J Anat* 1995; **186**: 639–644.
 21. Nguyen M, Shing Y, Folkman J. Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane. *Microvasc Res* 1994; **47**: 31–40.
 22. Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997; **277**: 48–50.
 23. Pepper MS, Sappino AP, Stocklin R, Montesano R, Orci L, Vassalli JD. Upregulation of urokinase receptor expression on migrating endothelial cells. *J Cell Biol* 1993; **122**: 673–684.
 24. Ziche M, Parenti A, Ledda F, *et al.* Nitric oxide promotes proliferation and plasminogen activator production by coronary venular endothelium through endogenous bFGF. *Circ Res* 1997; **80**: 845–852.
 25. Sato Y, Rifkin DB. Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J Cell Biol* 1988; **107**: 1199–1205.
 26. Mandriota SJ, Pepper MS. Vascular endothelial growth factor-induced *in vitro* angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. *J Cell Sci* 1997; **110**: 2293–2302.
 27. Wempe F, Lindner V, Augustin HG. Basic fibroblast growth factor (bFGF) regulates the expression of the CC chemokine monocyte chemoattractant protein-1 (MCP-1) in autocrine-activated endothelial cell. *Arterioscler Thromb Vasc Biol* 1997; **17**: 2471–2478.
 28. Bikfalvi A, Klein S, Pintucci G, Quarto N, Mignatti P, Rifkin DB. Differential modulation of cell phenotype by different molecular weight forms of basic fibroblast growth factor: possible intracellular signaling by the high molecular weight forms. *J Cell Biol* 1995; **129**: 233–243.
 29. Bonnet H, Filhol O, Truchet I, *et al.* Fibroblast growth factor-2 binds to the regulatory β subunit of CK2 and directly stimulates CK2 activity toward nucleolin. *J Biol Chem* 1996; **271**: 24 781–24 787.