



Original article

## Endogenous and exogenous fibroblast growth factor-2 modulate wound healing in the chick embryo chorioallantoic membrane

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### Abstract

Re-epithelization and the formation of a granulation tissue consisting of inflammatory cells, newly formed blood vessels, and fibroblasts embedded in a loose collagenous extracellular matrix, are critical events occurring during wound healing. In this study, utilizing the chick embryo chorioallantoic membrane (CAM) as an *in vivo* model of wound healing, we investigated the role of endogenous and exogenous fibroblast growth factor-2 (FGF-2) in the wound healing reparative processes. The results showed that: (1) neutralizing anti-FGF-2 antibodies (400 ng/embryo) decreased significantly the rate of wound healing (occurring only in 25% of specimens) when applied close to the edge of the wound, causing a significant decrease of microvessel and fibroblast density, and of an inflammatory macrophage infiltrate in the wounded area; (2) conversely, the application of exogenous recombinant FGF-2 (1.0 µg/embryo) greatly accelerated the wound repair occurring approximately 24 h earlier than in untreated CAMs, stimulating angiogenesis, fibroblast proliferation, and macrophage infiltration. These findings demonstrate the role of FGF-2 in wound healing of the CAM and suggest that CAM, usually employed as an *in vivo* assay to study angiogenesis, can also be utilized as an *in vivo* model for the easy, rapid, and economic screening of molecules potentially able to affect the wound healing process.

### Introduction

Wound healing is characterized by the formation of a granulation tissue consisting of inflammatory cells, newly formed blood vessels, and fibroblasts embedded in a loose collagenous extracellular matrix. Re-epithelization, angiogenesis and matrix deposition are critical events controlling this process [1].

Angiogenesis is confined to the wound site and plays a pivotal role for successful wound healing [2]. Indeed, revascularization is required to furnish the new tissue and to dispose the waste products of metabolism. Angiogenesis occurs as a highly regulated process, which is rapidly stimulated after injury and ceases when wound healing is complete [3]. Wound angiogenesis is believed to be initiated by the early release of preformed growth factors such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) [4, 5]. It has been recently suggested that FGF-2 and VEGF act

in concert in surgical wounds where the angiogenic stimulus is initiated by FGF-2 and is maintained by VEGF [6].

The chick embryo chorioallantoic membrane (CAM) is usually employed as an *in vivo* model to study angiogenesis and as a target for angiogenic and anti-angiogenic compounds [7]. Recently, we have utilized the CAM as *in vivo* model of wound healing [8]. Histological examination of the CAM during wound healing demonstrated hyperplasia of the chorionic epithelium in the area involved in the repair process and an inflammatory infiltrate consisting mainly of macrophages. Also, about three times as many microvessels and fibroblasts were present in the mesenchyme of the wounded area with respect to the adjacent control regions.

Previous observations have indicated the capacity of exogenous FGF-2 to accelerate wound healing in different experimental models [6, 9–13]. Also, endogenous FGF-2 has been demonstrated to exert a rate-limiting role in rat subcutaneous sponge models of wound repair [14, 15]. Recently, it has been demonstrated that the healing of excisional skin wounds is delayed in mice lacking FGF-2 [16]. Interestingly, FGF-2

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mRNA and protein are detectable in the CAM during chicken embryogenesis and experimental evidences point to a role for endogenous FGF-2 in the development of the CAM vasculature [17, 18]. On this basis, in the present paper we investigated the role of endogenous and exogenous FGF-2 on the reparative processes occurring in the CAM during wound healing.

## Materials and methods

### *Animals*

Fertilized White Leghorn chicken eggs were incubated under conditions of constant humidity at 37 °C. On the third day of incubation, a square window was opened in the egg shell after removal of 2–3 ml of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass of the same size and the eggs were returned to the incubator.

### *In vivo CAM wound assay*

At day 8 of incubation, a full thickness excision of a small (1 mm<sup>2</sup>) area of CAM devoid of large blood vessels was performed with the aid of a microknife under a Zeiss stereomicroscope SR (Zeiss, Oberkochen, Germany) equipped with the Camera System MC 63. In the experimental series, methylcellulose discs soaked with 400 ng/embryo of a rabbit polyclonal anti-FGF-2 antibody (kindly provided by Dr D.B. Rifkin, New York University Medical Center, New York, NY, USA) or with its controls, namely 1:10 normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) or with 1 µg/embryo of recombinant FGF-2 (Pharmacia, Milano, Italy) were placed near the margin of the lesion. For this purpose, the molecules were mixed with methylcellulose (0.25% final concentration) and discs were prepared by pipetting 10 µl of the mixture on sterile glass slides. Dried discs were implanted on top of the growing CAM. All procedures were performed under sterile conditions.

The eggs were then returned to the incubator and CAMs were examined daily until day 12 and photographed *in ovo*. At day 12, CAMs were fixed in Bouin's fluid, dissected in strips, embedded in paraffin, and serially sectioned at 7 µm for light microscopy.

### *Quantitation of the angiogenic response*

The microvascular density of the area close to the excision was evaluated by using a morphometric method of 'point counting' [19] and compared to the microvascular density of a distant control area in the same CAM, as control. Briefly, transversally cut microvessels (diameter ranging from 3 to 10 µm) were studied at 250× magnification with a square mesh inserted in the eyepiece of a Leitz Dialux 20 photomicroscope (Leitz,

Wetzlar, Germany). The mesh consisted of 12 lines per side, giving 144 intersection points. Six randomly chosen microscopic fields of each section (every third section within 30 serial slides from an individual specimen were analyzed) were evaluated for the number of intersection points occupied by microvessels. The microvessel density was expressed as the percentage of the total number of intersection points occupied by blood vessels.

### *Quantitation of macrophages*

After deparaffinization and rehydration, adjacent serial sections were stained for naphthol-AS-D-chloroacetate activity to highlight macrophages [20]. Then, macrophages were counted on 6 randomly selected 160× fields of every third section within 30 serial sections from each specimen in the CAM mesenchyme under the wound and compared to their number in a distant area from the wound, as control. The 144-point mesh described above was used and macrophage density was expressed as the percentage of intersection points occupied by naphthol-AS-D-chloroacetate activity-positive cells.

### *Quantitation of fibroblasts*

Fibroblasts, identified on the basis of their spindle-shaped morphology, were counted on 6 randomly selected 160× fields of every third section within 30 serial sections from each specimen in the CAM mesenchyme under the wound and compared to their number in a distant area from the wound, as control. The 144-point mesh was used and fibroblast density was expressed as the percentage of occupied intersection points.

### *Statistical analysis*

Means ± 1 standard deviation (SD) were determined for all variables. The statistical significance of the differences between mean values of the intersection points was determined by the Student's *t*-test for unpaired data.

## Results

### *Endogenous FGF-2 is implicated in wound healing of the chick CAM*

Previous observations had shown that a full-thickness 1 mm<sup>2</sup> excision of the CAM in an 8 day chick embryo causes a healing reaction characterized by a rapid vasoproliferative response [8]. In 75% of the embryos examined (*n* = 30), the solution of continuity was filled by a granulation tissue within 2 to 3 days after wounding, which developed a scar during the next 24–48 h. In contrast, when neutralizing anti-FGF-2 antibody (400 ng/embryo) was placed adjacent to the edge of the wound, closing of the solution of continuity was delayed until 6–7 days after wounding and occurred

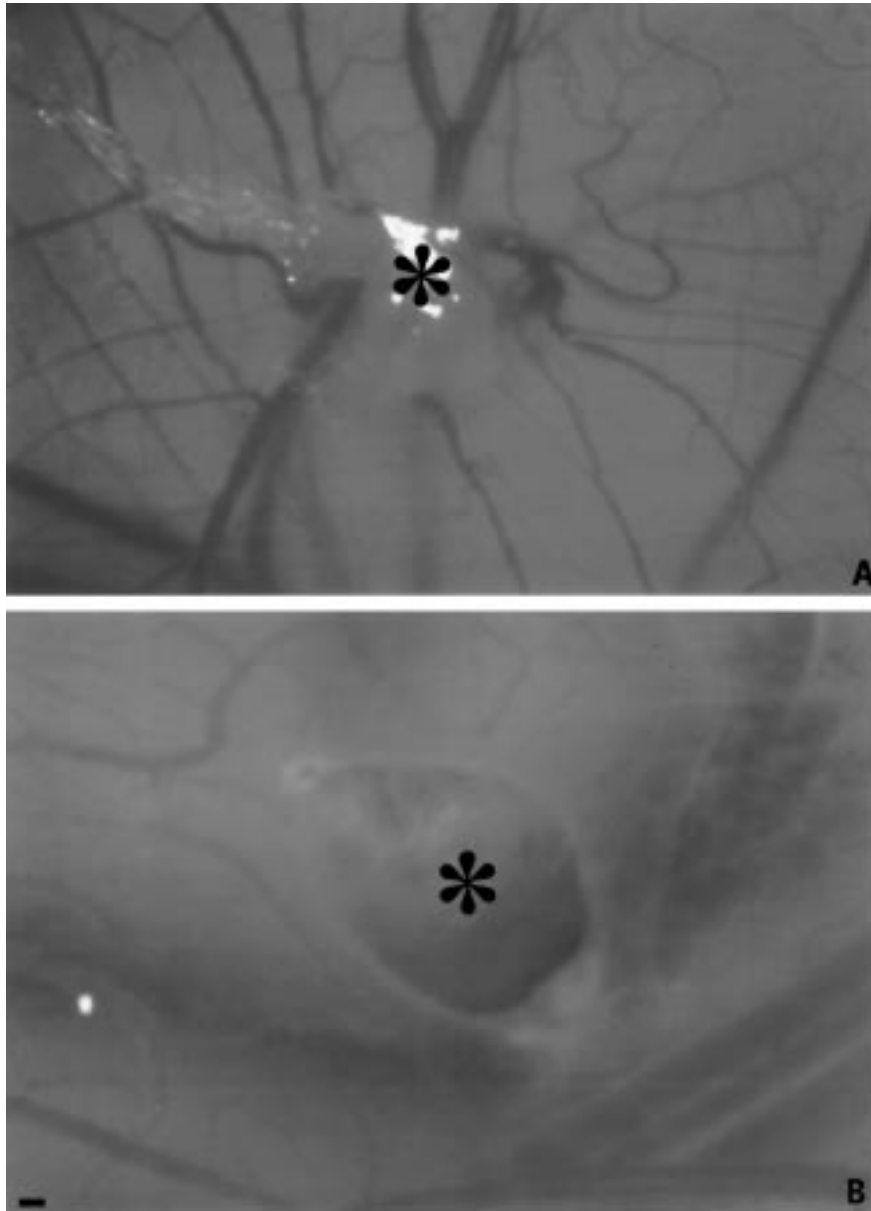


Figure 1. In (A), untreated CAM, 4 days after wounding. The solution of continuity has been filled by a scar tissue (asterisk). In (B), CAM treated with neutralizing anti-FGF-2 antibody (400 ng/embryo), 5 days after wounding. A solution of continuity (asterisk), smaller than the initial one, is still present. Bar = 0.3  $\mu$ m.

only in 25% of the specimens ( $n = 10$ ) (Figure 1). Control embryos treated with normal rabbit IgG or BSA displayed a wounding closure overlapping those of the untreated (Table 1).

Histologic examination of control CAMs, performed when the solution of continuity of the wound was filled by a granulation tissue, showed hyperplasia of the chorionic epithelium and a 3-fold increase of microvessel and fibroblast density in the underlying mesenchyme when compared to adjacent intact regions (Table 1; Figures 2A, B). The mesenchyme also showed the presence of a perivascular inflammatory infiltrate represented mainly by naphthol-AS-D-chloroacetate esterase-positive macrophages (Figure 2C). In contrast, a marked reduction of the angiogenic response and fibroblast density occurred in wounded CAMs treated

with anti-FGF-2 antibody (Table 1; Figure 2D). Also, the antibody prevented the formation of a significant macrophage infiltrate in the wounded area; these cells were located almost exclusively inside the vessels (Table 1; Figure 2E). Number of vessels, fibroblasts and macrophages under the wound was also significantly lower, as compared to their number in the adjacent control CAM regions (Table 1).

#### *Exogenous FGF-2 accelerates wound healing of the chick CAM*

When human recombinant FGF-2 (1.0  $\mu$ g/embryo) was placed near the margin of the lesion of the CAM immediately after wounding, the rate of wound healing was greatly accelerated and the formation of a scar

Table 1. Microvessel, fibroblast, and macrophage density in the CAM's intermediate mesenchyme under and distant from the wound.

	<i>n</i>	Treatment	Microvessels	Fibroblasts	Macrophages
Control CAM	10	–	4.2%	5.2%	2.1%
Wounded CAM	20	Untreated	12.4% under**	15.8% under**	5.2% under**
			5.2% distant	6.5% distant	2.0% distant
	20	Anti-FGF-2 Ab	2.1%* under**	3.2%* under**	1.0%* under**
			4.4% distant	5.4% distant	2.0% distant
	10	Rabbit IgG	11.2% under**	14.4% under**	4.8% under**
			5.4% distant	6.0% distant	2.3% distant
	10	BSA	12.5% under**	15.0% under**	5.0% under**
			5.0% distant	5.3% distant	2.1% distant
20	FGF-2	35.2%* under	45.7%* under	9.4%* under	
		6.0% distant	6.5% distant	2.4% distant	

Anti-FGF-2 antibody (400 ng/embryo), 1:10 normal rabbit IgG, 1% BSA, or human recombinant-FGF-2 (1 µg/embryo) were absorbed on methylcellulose discs and placed near to the margin of the lesion at day 8 of incubation. See 'Materials and methods' for further details.

\*  $P < 0.001$  vs untreated wounded CAM; \*\*  $P < 0.001$  vs distant counts.

occurred within 3–4 days, approximately 24 h earlier than in untreated CAMs (see above). Accordingly, microscopic examination of the CAMs revealed a 3-fold increase of microvessel and fibroblast density and a 2-fold increase of the density of infiltrating macrophages in the mesenchyme of FGF-2-treated CAMs with respect to untreated specimens (Table 1; Figures 2F, G). Number of vessels, fibroblasts and macrophages under the wound were also significantly higher, as compared to their number in the adjacent control CAM regions (Table 1).

## Discussion

Here we show that FGF-2 plays a key role in the reparative process of a full-thickness excision wound of the chick embryo CAM. The experimental evidence is twofold: (1) neutralizing anti-FGF-2 antibodies decrease significantly the rate of wound healing when applied close to the edge of the wound, causing a significant decrease of microvessel and fibroblast densities and of an inflammatory macrophage infiltrate in the wounded area; (2) conversely, recombinant FGF-2 greatly accelerates wound healing, stimulating angiogenesis, fibroblast proliferation, and macrophage infiltration. The developmental stage does not influence the time-course and the microscopic characteristics of the reparative process, which occur in the same way if the experiments start at day 12 of incubation, instead of day 8 (data not shown).

We have demonstrated previously that an Mr 16,000 FGF-2-like molecule is present in the CAM [17]. This molecule was identified as FGF-2 on the basis of its immunoreactivity, affinity for heparin, and capacity to induce a biological response in cultured endothelial cells. In a parallel series of experiments, the same neutralizing anti-FGF-2 antibody utilized in the present paper was able to exert a potent anti-angiogenic effect when applied onto the surface of the CAM [17, 18]. Anti-FGF-2 antibody also decreased fibroblast density, but did not affect epithelial cells of the chorion and allantois [17]. These findings pointed to a rate-limiting role for FGF-2 in the maturation of blood vessels and stroma

during CAM development, raising the possibility that endogenous chick FGF-2 may affect not only the proliferation of endothelial cells but also their migration, redistribution, and invasive behavior. *In situ* hybridization studies support this hypothesis [21]. Chorionic epithelial cells represent the major source of FGF-2 at early stages of development. FGF-2 released by these cells may induce an angiogenic response in undifferentiated vessels of CAM mesenchyme by a paracrine mode of action. At later stages of development, FGF-2 mRNA expression occurs mainly in endothelial cells of the capillary plexus, suggesting that at this time endogenous FGF-2 may play an autocrine role in endothelium. On this basis it could be anticipated that neutralizing anti-FGF-2 antibodies might deeply affect the wound healing process in the CAM. In our wound healing model, FGF-2 can be released from damaged cells [22] and/or can be mobilized from the extracellular matrix stores of the CAM by the action of several proteases found in early wounds [23, 24]. Our data support previous observations on the rate-limiting role of endogenous FGF-2 in rat subcutaneous sponge models of wound repair [14, 15], thus indicating that the involvement of FGF-2 in wound healing spans through different animal species.

Also, we have observed that exogenous application of FGF-2 accelerates wound healing in the chick embryo CAM, as already reported for different wound healing models in rodents [10, 25] and humans [26].

Taken together our observations raise the question about the biological response(s) triggered by endogenous and exogenous FGF-2 after wounding and responsible for the observed effects on the rate of wound healing. Wound healing is accelerated by exogenous angiogenic factors [27–29]. Conversely, wound healing is retarded when neovascularization is suppressed or delayed [14, 30]. Thus, endogenous and exogenous FGF-2 may affect wound healing in the CAM by modulating endothelial cell behavior. However, various cell types besides endothelial cells carry tyrosine-kinase FGF receptors [31]. Accordingly, in parallel with the modifications in vascular density observed in wounded

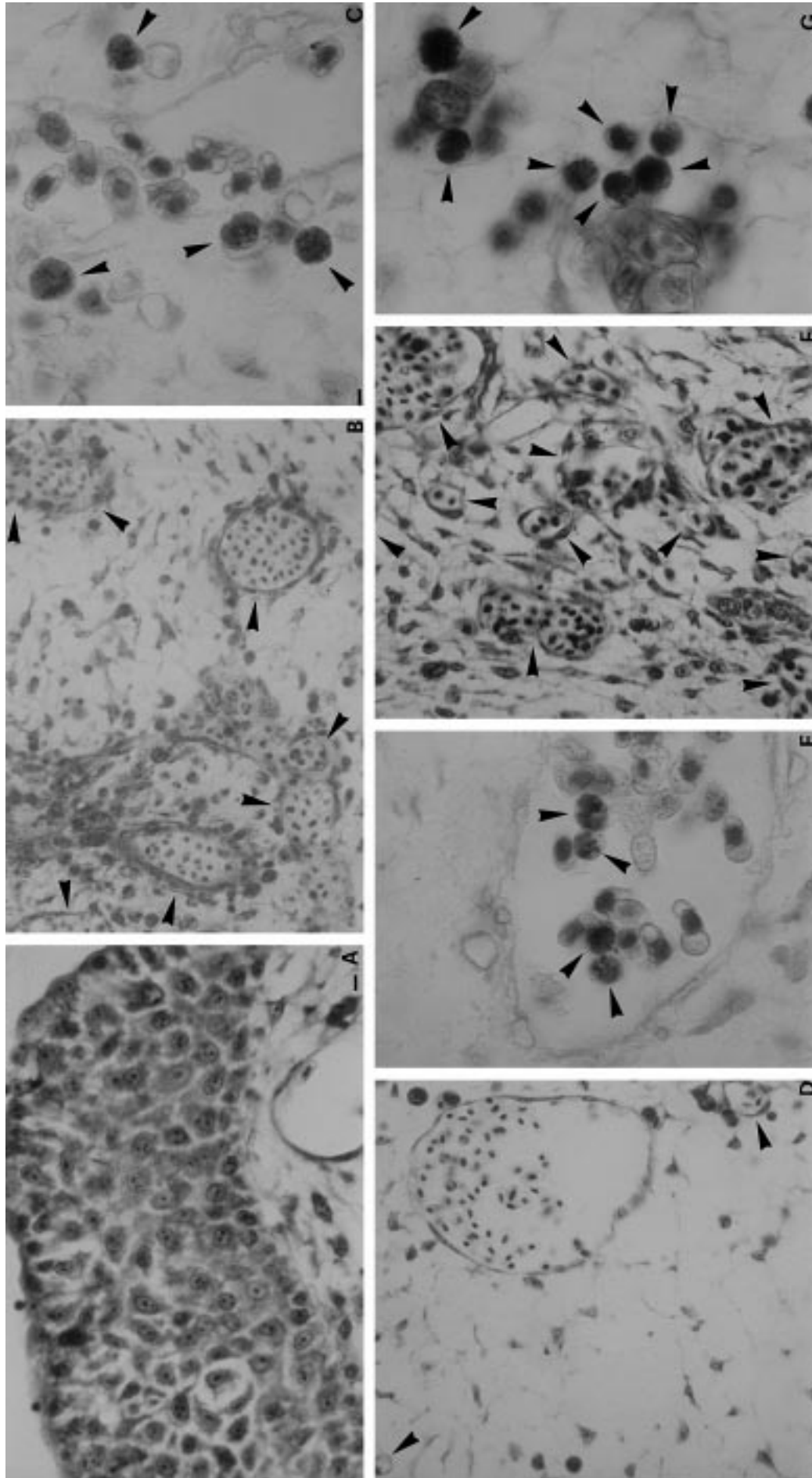


Figure 2. (A-C) Light micrographs of an untreated CAM, 4 days after wounding. Note in (A), the hyperplasia of the chorionic epithelium; in (B), numerous vessels (arrowheads), fibroblasts and mononuclear cells in the CAM mesenchyme and in (C), naphthol-AS-D-chloroacetate esterase positive cells (arrowheads) in perivascular position. (D, E) Light micrographs of a CAM, treated with neutralizing anti-FGF-2 antibody, 6 days after wounding. Note in (D) two microvessels (arrowheads), scarce fibroblasts and in (E), of naphthol-AS-D-chloroacetate esterase positive cells (arrowheads) inside a vessel. (F, G) Light micrographs of a CAM, treated with human recombinant-FGF-2, 3 days after wounding. Note in (F) a higher number of vessels (arrowheads), fibroblasts and mononuclear cells and in (G) of naphthol-AS-D-chloroacetate esterase positive cells (arrowheads), in perivascular position. Bar = 0.025  $\mu\text{m}$  (A, B, D, F); 0.01  $\mu\text{m}$  (C, E, F).

CAMs treated with anti-FGF-2 antibody or recombinant FGF-2, we have demonstrated congruent modifications in fibroblast density and macrophage infiltrate. Indeed, antibody treatment caused a significantly decrease in the number of microvessels, fibroblasts and of macrophage infiltrate. Conversely, FGF-2 administration increased all the parameters tested.

Recently, the transformation of fibroblasts into endothelial cells has been demonstrated during angiogenesis [32]. Moreover, co-cultures of endothelial cells and fibroblasts elicited an angiogenic response in collagen gels as demonstrated by the reorganization of endothelial cells into a capillary-like network [33, 34]. Accordingly, the capacity of primary fibroblasts to stimulate angiogenesis has been reported [35, 36]. Also, several lines of evidence point to tissue macrophages as key contributors in angiogenesis during wound healing. Macrophages migrate in the depth of a wound where oxygen tension is very low [37], a condition stimulating these cells to release angiogenic factors, including FGF-2, VEGF, transforming growth factor- $\alpha$  and - $\beta$ , granulocyte-macrophage colony stimulating factor, interleukin-8, and tumor necrosis factor- $\alpha$  [38, 39]. It is to note that wound healing is delayed when transformation of monocytes into macrophages is prevented by corticosteroids [40]. FGF-2 is chemotactic for macrophages [41] and stimulates endothelium to release the macrophage chemoattractant chemokine MCP-1 [42]. Taken together, these data indicate that FGF-2 can deeply affect the cross-talk among endothelial cells, fibroblasts, and macrophages during wound healing.

In conclusion, our findings demonstrate the role of FGF-2 in wound healing of the CAM. Also, the data confirm the possibility to utilize the chick embryo CAM as an *in vivo* model for the easy, rapid, and economic screening of molecules potentially able to affect the wound healing process.

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