

Transforming Growth Factor β 1-induced Changes in Cell Migration, Proliferation, and Angiogenesis in the Chicken Chorioallantoic Membrane

Edmund Y. Yang and Harold L. Moses

Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, 37232

Abstract. Application of TGF β 1 (10–100 ng) to the chicken chorioallantoic membrane (CAM) for 72 h resulted in a dose-dependent, gross angiogenic response. The vascular effects induced by TGF β 1 were qualitatively different than those induced by maximal doses of basic FGF (bFGF) (500 ng). While TGF β 1 induced the formation of large blood vessels by 72 h, bFGF induced primarily small blood vessels. Histologic analysis revealed that TGF β 1 stimulated pleiotropic cellular responses in the CAM. Increases in fibroblast and epithelial cell density in the area of TGF β 1 delivery were observed as early as 4 h after TGF β 1 treatment. By 8 h, these cell types also demonstrated altered morphology and marked inhibition of proliferation as evidenced by ^3H -thymidine labeling. Thus, the TGF β 1-stimulated accumulation of these cell types was the result of cellular chemotaxis

from peripheral areas into the area of TGF β 1 delivery. Microscopic angiogenesis in the form of capillary sprouts and increased endothelial cell density first became evident at 16 h. By 24 h, capillary cords appeared within the mesenchyme of the CAM, extending towards the point of TGF β 1 delivery. ^3H -thymidine labeling revealed that the growth of these capillary cords was due to endothelial cell proliferation. Finally, perivascular mononuclear inflammation did not become evident until 48 h of treatment, and its presence correlated spatially and temporally with the gross and histological remodelling of newly formed capillary cords into larger blood vessels. In summary, these data suggest that, in the chicken CAM, TGF β 1 initiates a sequence of cellular responses that results in growth inhibition, cellular accumulation through migration, and microvascular angiogenesis.

TRANSFORMING growth factor beta (TGF β)¹ was originally identified by its ability to induce the reversible transformation of nonneoplastic cells in culture (Moses et al., 1981; Roberts et al., 1981). Thus, it was initially implicated to have a role in the process of neoplastic transformation. Subsequently, however, TGF β was identified in many normal tissues (Proper et al., 1982; Roberts et al., 1982), additional TGF β biologic activities were discovered (reviewed in Moses et al., 1988), and several growth factors closely related to TGF β were identified (TGF β 1, TGF β 2, and TGF β 3; reviewed in Lyons and Moses, 1990). Our concepts of the roles of TGF β in vivo have therefore evolved. The growth factors comprising the TGF β family are now thought to have roles in embryological development and normal physiology, as well as in pathophysiology.

Our best understanding of TGF β function in vivo is in wound healing. TGF β is stored in platelets (Childs et al., 1982) and released from platelets during degranulation (Assoian and Sporn, 1986). TGF β is also produced by cultured

T-cells (Kehrl et al., 1986), macrophages (Assoian et al., 1987), monocytes (Assoian et al., 1987), neutrophils (Grotendorst et al., 1989), and fibroblasts (Tucker et al., 1983; Pircher et al., 1984). These cell types have important, integrated functions in wound healing, and hence it is not surprising that TGF β is a biologically significant component of wound fluid derived from subcutaneous wound chambers (Cromack et al., 1987; Grotendorst et al., 1988). Additionally, in vivo administration of TGF β 1, the prototypic TGF β , yields a wound healing response. When TGF β 1 is injected subcutaneously into newborn mice a granulation tissue response characterized by inflammation, fibrosis, and angiogenesis is observed (Roberts et al., 1986). TGF β 1 can stimulate increased tensile strength of incisional wounds (Mustoe et al., 1987) and reverse the wound healing deficits induced by glucocorticoids (Pierce et al., 1989a) and adriamycin (Lawrence et al., 1985). Thus, although the specific inhibition of endogenous TGF β has not yet been shown to impair normal wound healing, TGF β is most likely a natural mediator of wound healing.

To understand the mechanisms by which TGF β might function in wound healing and other processes, the effects of TGF β have been studied on isolated cells in vitro. These

1. *Abbreviations used in this paper:* bFGF, basic FGF; CAM, chicken chorioallantoic membrane assay; PMN, polymorphonuclear cell; SEC, subepithelial capillary network; TGF β , transforming growth factor beta.

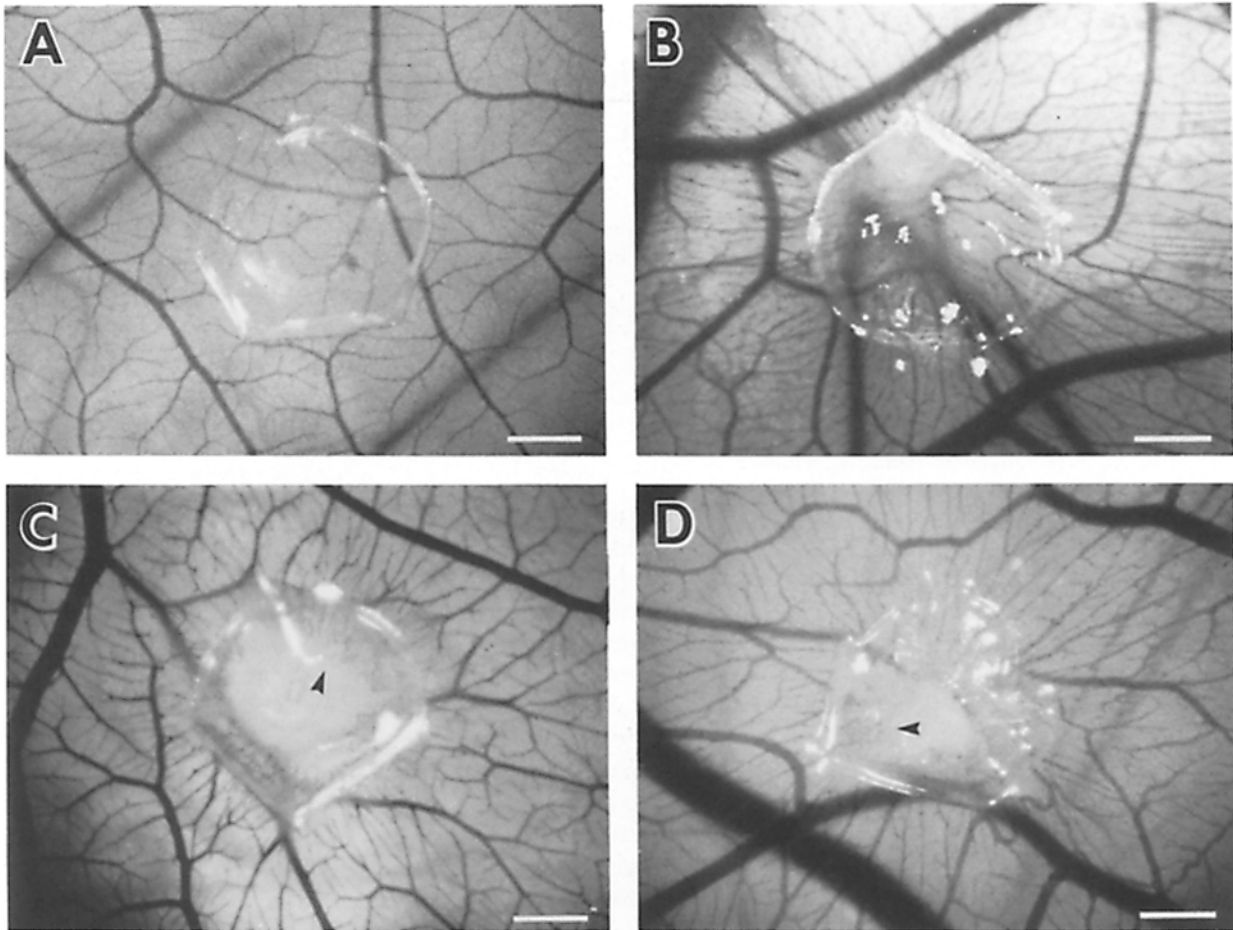


Figure 1. CAM vascular reactions to TGF β 1 and bFGF. A control methylcellulose disk (A) and methylcellulose disks impregnated with either 50 ng TGF β 1 (B and D) or 500 ng bFGF (C) were placed onto the CAM for 72 h (A-C) or 24 h (D). TGF β 1 induced a marked angiogenic response after 72 h (B) as compared to the control (A). Relative to the small blood vessels induced by bFGF at 72 h (arrowhead, C), TGF β 1 consistently induced the formation of much larger blood vessels. This was the result of vascular remodeling since much smaller blood vessels were induced by TGF β 1 after only 24 h (arrowhead, D). Bars, 1 mm.

studies have demonstrated that many of the tissue responses associated with in vivo TGF β 1 administration result from the direct action of TGF β 1 on specific cell types (reviewed in Roberts et al., 1988; Lyons and Moses, 1990). However, in vitro studies on endothelial cells have not yielded a clear understanding of the mechanisms by which TGF β induces angiogenesis. The direct effects of TGF β 1 on cultured endothelial cells are perplexing, perhaps because the many in vitro endothelial cell assays model different aspects of angiogenesis. In vitro assays that measure the morphologic reorganization of individual endothelial cells into capillary tubes (in vitro angiogenesis) model the later stages of angiogenesis when loosely connected capillary endothelial cells differentiate from a proliferative, migratory phenotype into a quiescent, vascular epithelium phenotype (Wagner, 1980). TGF β 1 is stimulatory to this process. When endothelial cells are dispersed in three-dimensional collagen gels, TGF β 1 stimulates their morphologic reorganization into capillary tube structures and stimulates cell-mediated contraction of the collagen gel (Madri et al., 1988). This suggests that TGF β 1 may be important in directly stimulating the resolution of angiogenesis or in maintaining the differentiated state of endothelial cells.

In vitro endothelial cell assays that measure endothelial cell protease production, invasion, migration, or proliferation model early events in angiogenesis (Folkman, 1985). TGF β 1 is inhibitory in these assays. TGF β 1 inhibits endothelial cell production of plasminogen activator (Saksela et al., 1987), and correspondingly, it inhibits endothelial cell invasion of human amniotic membranes (Mignatti et al.,

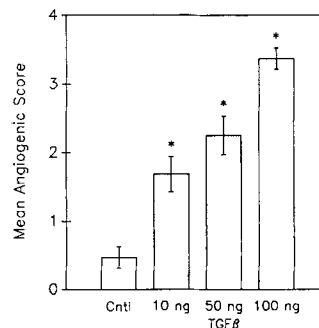


Figure 2. Dose-responsive effect of TGF β 1 on mean angiogenic scores. Control methylcellulose disks (Cntl) and methylcellulose disks impregnated with either 10, 50, or 100 ng of TGF β 1 were applied to the CAM for 72 h. Vascular responses for each disk were then scored on a 0-4+ scale based on the criteria described in Materials and Methods. Each value is the mean \pm SEM, $n \geq 16$. (*) $P < 0.001$ as compared to control.

1989). TGF β 1 is also inhibitory to endothelial cell migration and proliferation (Müller et al., 1987); even submaximal inhibitory doses cannot be overcome by the endothelial cell mitogens acidic or basic FGF (bFGF) (Frater-Schroder et al., 1986; Baird and Durkin, 1986). Furthermore, when endothelial cells are plated on the surface of collagen gels, TGF β 1 inhibits PMA-induced endothelial cell invasion and subsequent capillary tube formation (Müller et al., 1987).

Taken collectively, the direct effects of TGF β 1 on endothelial cells in vitro might suggest that TGF β 1 is a general repressor of angiogenesis. Yet, administration of TGF β 1 in vivo yields endothelial cell growth and the formation of new capillaries, a definitive angiogenic response (Roberts et al., 1986). To reconcile these paradoxical observations, we have used the chicken chorioallantoic membrane (CAM) assay to examine the temporal and spatial sequence of cellular responses that occurs during TGF β 1-induced angiogenesis.

Materials and Methods

Embryos and Reagents

Fertilized white leghorn chicken eggs were obtained from Truslow Farms (Chestertown, MD). Porcine TGF β 1 was obtained from R&D Systems (Minneapolis, MN). Recombinant human bFGF was the kind gift of Dr. Lawrence Coussens of Chiron Corporation (Emeryville, CA).

CAM Assay

The methods used for egg incubation have been described (Auerbach et al., 1974). Methylcellulose disks were prepared through the dehydration of methylcellulose solutions on teflon rods as described (Taylor and Folkman, 1982). Porcine TGF β 1 was dissolved in filter-sterilized 4 mM HCl, 0.5% BSA at a concentration of 10 ng/ml. Recombinant human bFGF was dissolved in filter-sterilized 10 mM Tris-HCl, 1 mM EDTA, pH 7.4, at a concentration of 0.5 mg/ml. Growth factors, BSA (used as a carrier), and deionized water were added to 1% methylcellulose to yield the appropriate concentration of growth factor in 0.5% methylcellulose, 1.25 mg/ml BSA. All solutions were sterile. For control disks the hydration buffer without growth factor was added.

Methylcellulose disks were placed onto the CAMs on day 9 of incubation and after 3 d the angiogenic responses to each disk were scored without knowing their growth factor composition. Scores were based on a 0–4+ response scale as previously described (Vu et al., 1985). After all disks were scored, their compositions were revealed, and the mean angiogenic score for each treatment was compared to the mean score for the control disks using the two-tailed *t* test.

Histology and Autoradiography

After scoring, CAMs were flooded with Bouin's fixative in situ and additional fixative was injected into the allantoic sac. Tissue samples were removed from the CAM, dehydrated through alcohol, and embedded in paraffin wax. 10- μ m sections were mounted on slides and stained with hematoxylin and eosin. Morphologic criteria were used to identify specific cell types. Endothelial cells were identified by their localization surrounding vascular lumens and their flat, epithelial appearance. Inflammatory cells were identified through their distinctive granular cytoplasm (polymorphonuclear cells) or enlarged size (mononuclear cells) (Lucas, 1974).

For autoradiography, 20 μ l of 3 H-thymidine, 1.0 mCi/ml (\approx 80 Ci/mmol; New England Nuclear, Boston, MA) in PBS, was pipetted onto each methylcellulose disk and the surrounding areas. After a 4-h incubation, the CAMs were carefully overlaid with Bouin's fixative and prepared for histology as described above. 10- μ m sections were deparaffinized and then coated with 1:1 water-diluted Ilford K-5 emulsion (Polysciences, Inc., Warrington, PA) for autoradiography. At the appropriate times, slides were developed in 1.6% Kodak D-19 developer and fixed in 30% sodium thiosulfate. The sections were then counterstained with toluidine blue.

To calculate endothelial cell labeling indices, representative autoradiography sections from control or TGF β 1-treated CAM tissue samples were ex-

amined for total endothelial cell numbers and total 3 H-thymidine labeled endothelial cells. Over 100 endothelial cells were examined per tissue sample. The resultant endothelial cell labeling indices were statistically analyzed using the two-tailed *t* test.

Morphometric and Cell Density Measurements

Multiple CAM tissue samples of a given time point were fixed and embedded. From each tissue sample serial sections were made, representative sections were chosen, and the sections were analyzed using a computerized morphometric analysis program (Southern Micro Instruments, Inc., Atlanta, GA).

To calculate mean CAM thickness, all of the representative sections from a particular tissue sample were measured for maximal thickness and averaged to yield a mean CAM thickness for that tissue sample. The mean CAM thickness measurements for all of the tissue samples of a given time point were then averaged to yield a mean CAM thickness for that time point.

For calculating fibroblast and endothelial cell densities, areas of known size excluding blood vessels larger than 50 μ m were demarcated. All of the fibroblasts and endothelial cells within the demarcated areas were counted and cell densities calculated. The cell densities from the representative sections of each tissue sample were averaged to yield the mean cell densities for each tissue sample. Then, the mean cell densities for all tissue samples of a given time point were averaged to yield the mean cell densities for each time point. A similar analysis was utilized for calculating mean epithelial cell densities, except that densities were calculated per unit length of the CAM instead of per unit area.

Results

Gross Vascular Effects Induced by TGF β 1

To examine the effects of TGF β 1 on the CAM vasculature TGF β 1-impregnated methylcellulose disks were used as a local delivery system. This system has been used previously for the delivery of bFGF, protamine, heparin, and steroids to the CAM (Shing et al., 1985; Taylor and Folkman, 1982; Folkman et al., 1983). Application of a control methylcellulose disk to the CAM for 72 h resulted in little or no change in the normal blood vessel pattern (Fig. 1 *a*). However, application of a TGF β 1-impregnated methylcellulose disk (50 ng) for the same period of time resulted in a marked angiogenic effect (Fig. 1 *b*). TGF β 1 stimulated an increase in the density of arterioles and venules arranged in a radial pattern around the disk.

The angiogenic effects were scored on a 0–4+ scale based on the morphologic appearance of the vascular responses (see Materials and Methods for scoring system). Application of 10, 50, or 100 ng of TGF β 1 to the CAM resulted in a dose responsive increase in the mean angiogenic score (Fig. 2). All three doses resulted in a significantly higher mean score over control disks ($P < 0.001$, two-tailed *t* test).

Since the CAM assay has been used extensively for the testing of bFGF-induced angiogenesis (Gospodarowicz et al., 1985; Shing et al., 1985), we compared the vascular effects induced by bFGF with those of TGF β 1. A maximally effective dose of bFGF (500 ng or higher) induced angiogenic effects that were qualitatively different than the effects induced by TGF β 1. After 72 h, when the vascular effects were greatest, bFGF induced the growth of very small blood vessels (Fig. 1 *c*), as compared to the larger vessels induced by TGF β 1 (Fig. 1 *b*). Because the mechanism of TGF β 1-induced angiogenesis could be through the production of bFGF, we examined TGF β 1-treated CAMs at earlier times to determine if the vascular changes were similar to those induced by bFGF. Angiogenesis was first grossly discerned at 24 h after TGF β 1 administration (Fig. 1 *d*). Although small

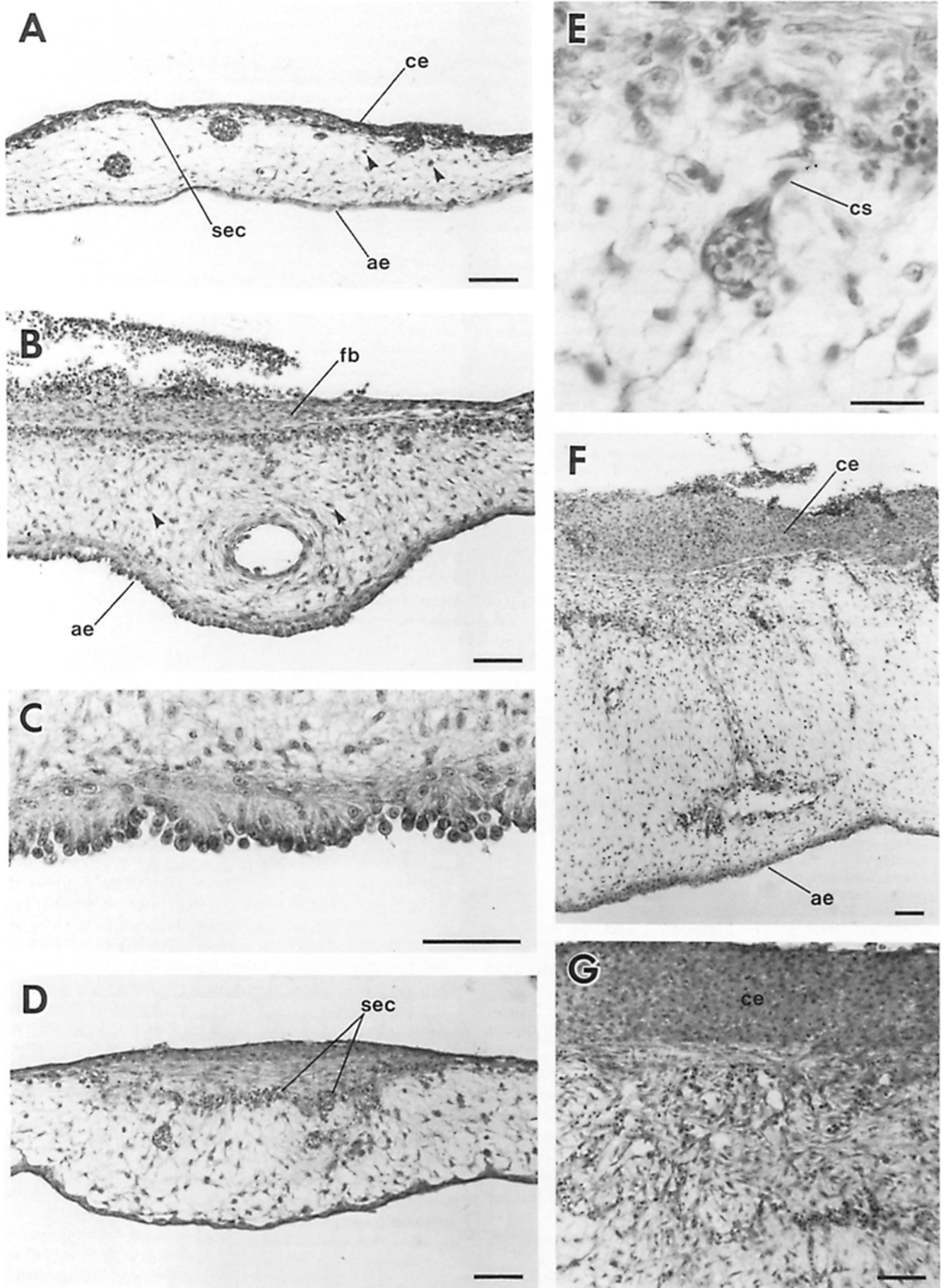


Figure 3.

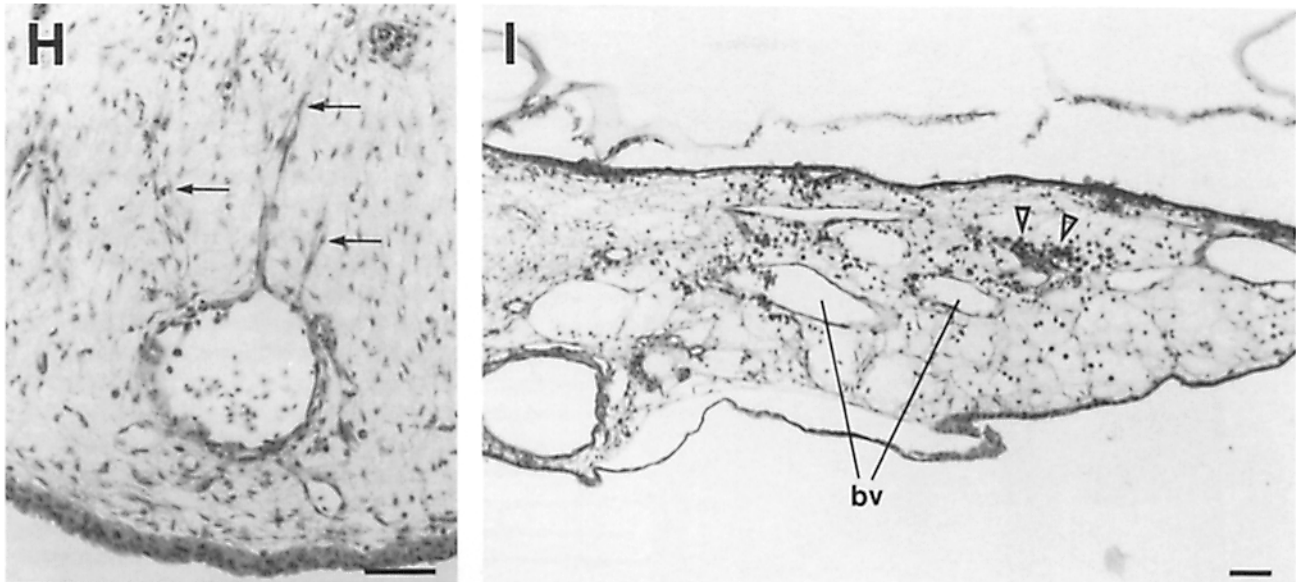


Figure 3. Histologic effects induced by TGF β 1 at various times after treatment. (A) Control-treated CAMs harvested at 72 h demonstrated thin chorionic and allantoic epithelia (*ce* and *ae*), sparse fibroblasts, and an SEC (*sec*) juxtaposed to the chorionic epithelium. Occasional PMNs (*closed arrowheads*) were observed. (B) CAMs treated with TGF β 1 (50 ng) for 8 h demonstrated fibroblast (*fb*) accumulation between the SEC and the chorionic epithelium, allantoic epithelial thickening, and accumulated sloughed cells on the surface of the chorionic epithelium. (C) After 8 h of TGF β 1 treatment, the allantoic epithelium demonstrated thickening due to cellular hypertrophy and acquisition of columnar morphology. (D) Increased numbers of capillaries were first visible at 16 h in the SEC and in the mesenchyme of the CAM. (E, higher magnification of D) Occasionally capillary sprouts (*cs*) were observed. (F) At 24 h, both chorionic and allantoic epithelial stratification were apparent. (G) Also at 24 h, endothelial cells were observed to invade the fibroblasts that had accumulated above the SEC, forming a granulation-like tissue (compare to Fig. 4 *d*). (H) Furthermore, fibroblasts as well as capillary cords (*arrows*) demonstrated radial organization relative to the point of TGF β 1 delivery. (I) At 72 h, mononuclear infiltration was evident (*open arrowheads*) in a perivascular distribution (*bv*, blood vessel). Representative sections (10 μ m) were stained with hematoxylin and eosin. Bars: (A–D, F–I) 50 μ m; (E) 20 μ m.

blood vessels were apparent, they converged upon a central, opaque area which, as shown below, corresponded to an area of increased fibroblast density and epithelial thickening. Between 24 and 72 h the TGF β 1-induced angiogenesis appeared to remodel from very fine blood vessels to larger vessels (compare Fig. 1, *b* and *d*). Neither the central opacity visualized at 24 h, nor the apparent vascular remodeling between 24 and 72 h were observed after bFGF administration.

Microscopic Effects Induced by TGF β 1

The progression of TGF β 1-induced angiogenesis was then examined histologically. Serial cross sections were made through samples of CAMs treated with TGF β 1 (50 ng) for varying times, and central sections were examined for cellular responses. Consistent with the gross observations, 72-h control-treated CAMs were of uniform thickness both beneath the methylcellulose disk and in the peripheral regions. From 0 to 72 h, the histologic appearance of control-treated CAMs was exactly the same. CAMs that had been treated with control methylcellulose disks for 72 h (Fig. 3 *a*) demonstrated a thin chorionic epithelium, a very fine subepithelial capillary network (SEC) just under the chorionic epithelium, a mesenchymal layer consisting of sparse fibroblasts and occasional blood vessels, and a very thin allantoic epithelium. The control methylcellulose disks did not cause wounding or inflammation.

In contrast, application of TGF β 1-impregnated methylcellulose disks (50 ng) to the CAM resulted in alterations in fibroblast, epithelial, and endothelial cell morphology. After

an 8-h exposure to TGF β 1, fibroblasts beneath the SEC appeared to accumulate to a higher density, and they demonstrated a distinctive bipolar morphology with vertical alignment relative to the plane of the CAM (Fig. 3 *b*). Fibroblasts were also observed to accumulate above the SEC, aligning parallel to the plane of the CAM and forcing the SEC to move downwards (Fig. 3 *b*; compare Fig. 3, *d* and *a*). The fibroblasts beneath the SEC expressed increased amounts of fibrillar, extracellular matrix-like material which was probably composed partially of collagen since these fibrils stained positive with Masson-Trichrome stain (data not shown). Both allantoic and chorionic epithelial thickening were also observed by 8 h. The chorionic epithelium thickened via stratification (Fig. 3 *d*), and the allantoic epithelium thickened primarily via cellular hypertrophy and the acquisition of columnar morphology (Fig. 3 *c*). These epithelial effects were not always apparent in the same sections since they were not always parasagittal. Polymorphonuclear cell (PMN) inflammation was evident by 8 h, but the distribution of these cells was fairly restricted to the peripheral regions. Within the centrally affected areas few inflammatory cells were noted. An accumulation of necrotic cells was usually observed between the TGF β 1-impregnated methylcellulose disk and the allantoic epithelium (e.g., Fig. 3 *b*). These necrotic cells were primarily composed of sloughed chorionic epithelial cells, but RBCs and PMNs could also be found.

Increased numbers of capillaries were first apparent at \sim 16 h after TGF β 1 treatment. Capillaries within the SEC appeared to be more numerous and more densely packed at this

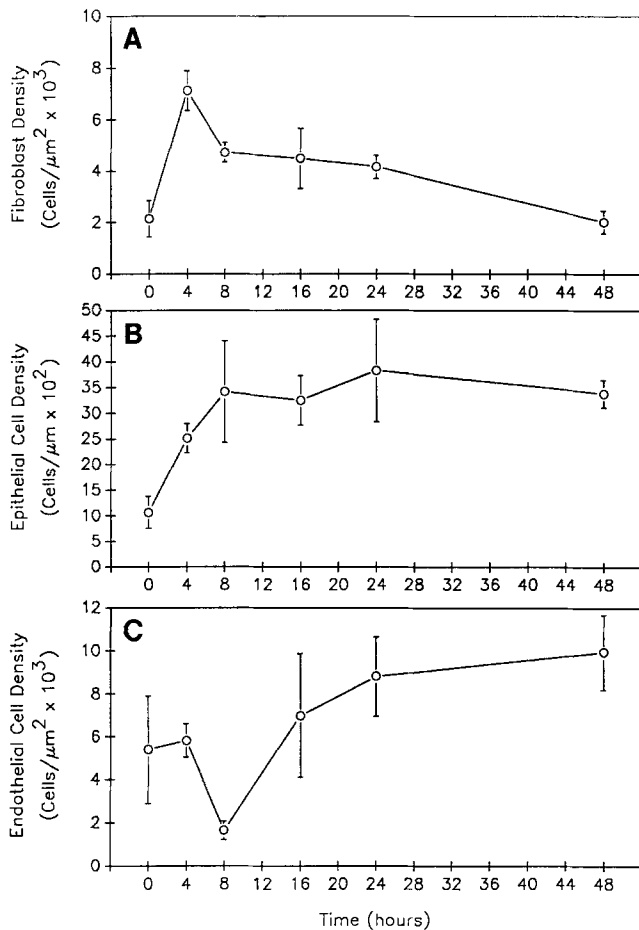


Figure 4. Effects of TGF β 1 on CAM cell densities. (A) Fibroblast; (B) allantoic epithelial cell; and (C) endothelial cell densities were measured at various times after TGF β 1 (50 ng) treatment. Each point represents the mean cell density \pm SEM of at least three tissue samples. Fibroblast and endothelial cell densities are expressed per unit area, whereas allantoic epithelial cell density is expressed per unit length of the CAM. See Materials and Methods for detailed description of cell density calculations.

time (Fig. 3 *d*). Small blood vessels beneath the SEC also appeared more numerous, and they revealed occasional capillary sprouts extending towards the SEC (Fig. 3 *e*). Neither mononuclear nor PMN inflammatory cells were associated with these capillary sprouts.

Over the course of 24 h the area of TGF β 1-induced effects enlarged and earlier responses were amplified. 24-h TGF β 1-treated CAMs were \sim 4-fold thicker as compared to controls ($P < 0.0001$, $n = 8$, two-tailed *t* test) and demonstrated an enlarged area of epithelial and fibroblastic effects. Both the allantoic and chorionic epithelia became markedly stratified (Fig. 3 *f*). Much of the fibroblast accumulation seen beneath the SEC at 8 h had diminished by 24 h (compare fibroblast densities in Fig. 3, *h* and *b*). However, fibroblasts continued to accumulate above the SEC at the mesenchymal/epithelial boundary (Fig. 3 *f*), and endothelial cells lining small capillaries were also seen to invade this space forming a granulation-like tissue (Fig. 3 *g*). The central location of the epithelial cell stratification and fibroblast accumulation probably

accounts for the grossly opaque appearance of the CAM at 24 h (e.g., Fig. 1 *d*).

At 24 h, fibroblasts beneath the SEC were aligned radially relative to the point of TGF β 1 delivery (Fig. 3 *f*). This radial pattern was reiterated in the developing capillary cords which extended from larger blood vessels towards the point of TGF β 1 delivery (Fig. 3, *f* and *h*). While a few of these capillary cords traversed the full distance between the larger blood vessels and the SEC (e.g., Fig. 3 *f*), most appeared incomplete and lacked connection with the SEC (Fig. 3 *h*). This was determined by examining serial sections in many different TGF β 1-treated CAM tissue samples. Inflammatory cells were not associated with these capillary cords.

From 24 to 72 h the fibroblast and epithelial hypercellularity receded, yet the gross and histologic angiogenesis persisted. By 48 h the CAM appeared to become thinner and fewer fibroblasts were apparent. Significant mononuclear inflammation was first noted at this time, particularly near blood vessels (data not shown). At 72 h, fibroblast numbers were diminished and mononuclear cell inflammation continued to accumulate (Fig. 3 *i*), yet focal concentrations of large blood vessels were clearly evident (Fig. 3 *i*). Since the gross angiogenic effect stimulated by TGF β 1 was observed to remodel from small vessels to larger vessels between 24 and 72 h (Fig. 1, *d* and *b*), the corresponding histologic changes in blood vessel size and numbers compare well (Fig. 3, *f* and *i*).

Effect of TGF β 1 on Cell Density

TGF β 1 has been shown to affect both cellular chemotaxis and proliferation. To separate the effects of TGF β 1 on these two processes in the CAM, the changes in cellular density and ³H-thymidine incorporation after TGF β 1 treatment were examined. As mentioned previously, from 0 to 72 h control-treated CAMs did not demonstrate any changes in gross or histological appearance. The fibroblast, epithelial cell, and endothelial cell densities in control-treated CAMs were constant during this period of time. Initial observations suggested that two populations of fibroblasts developed after TGF β 1 (50 ng) treatment. Fibroblasts above the SEC at the mesenchymal/epithelial border appeared to accumulate to the highest density (e.g., Fig. 3 *g*), whereas fibroblasts beneath the SEC appeared to accumulate only transiently at \sim 8 h after TGF β 1 treatment (e.g., Fig. 3 *b*). Due to the difficulty in distinguishing fibroblasts from endothelial cells above the SEC, only fibroblasts beneath the SEC and in the central area of TGF β 1 effect were counted. The density of this population of fibroblasts increased \sim 3.5-fold during the first 4 h of TGF β 1 treatment, then slowly decreased over 48 h to control levels (Fig. 4 *a*). Within the centrally affected area, the density of allantoic epithelial cells reached a plateau level \sim 3-fold higher than controls at 8 h of treatment (Fig. 4 *b*). Although the density of allantoic epithelial cells remained elevated at 48 h, by 72 h it was very close to control levels (e.g., Fig. 3 *i*).

Endothelial cells demonstrated a different temporal pattern of density regulation (Fig. 4 *c*). Only endothelial cells lining small blood vessels (<50 μ m diameter) and capillaries beneath the SEC were included in density measurements since endothelial cells within the SEC could not be adequately differentiated from fibroblasts. While the densities of fibroblasts and epithelial cells increased over the first 4–8 h,

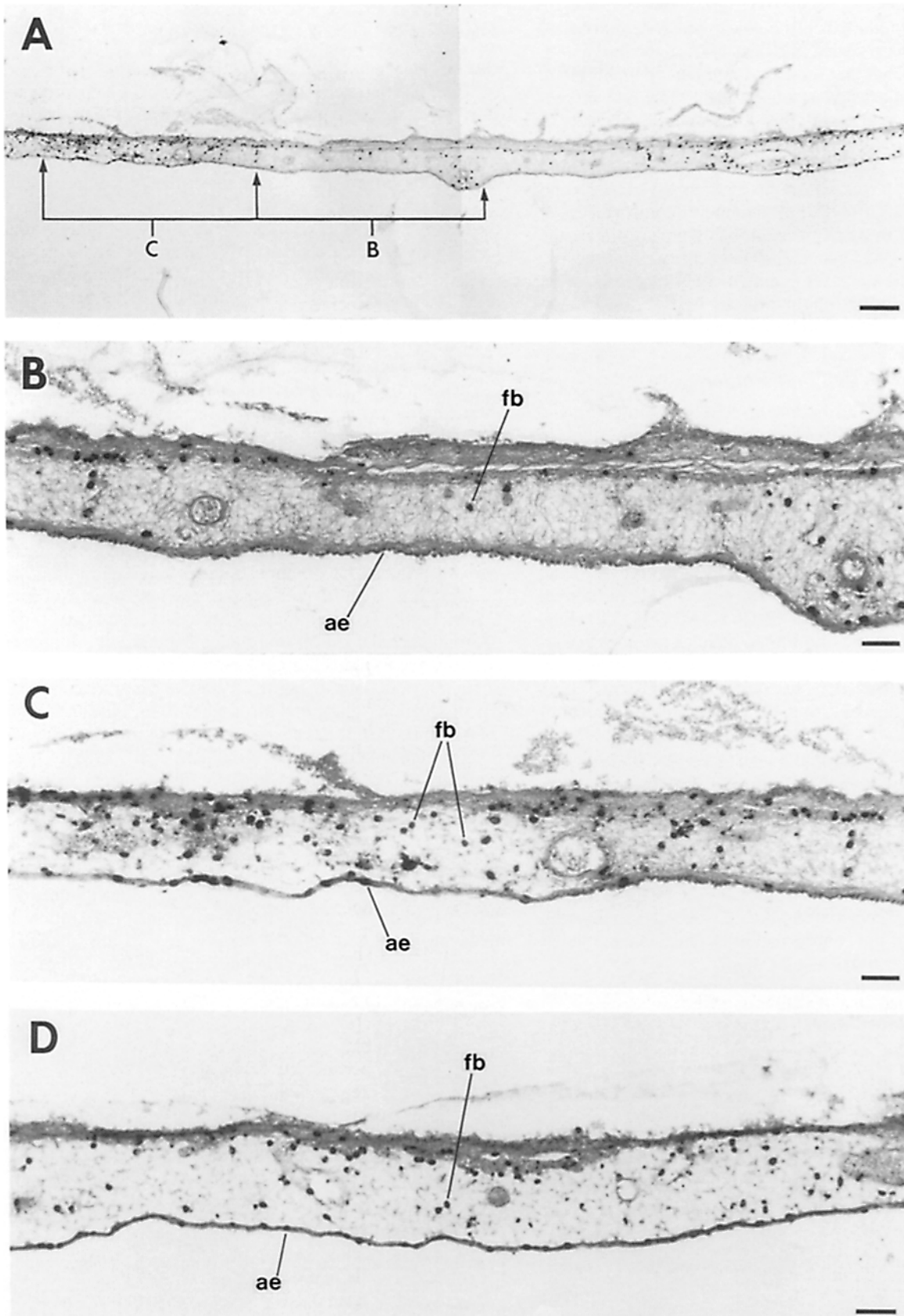


Figure 5. CAM cellular proliferation after 8 h TGF β 1 treatment as revealed by ^3H -thymidine labeling from 4 to 8 h. (A) CAMs treated with TGF β 1 (50 ng) for 8 h demonstrated a central zone of decreased cellular labeling with peripheral areas of increased labeling. B and C represent areas shown at higher magnification below. (B) High power magnification of central zone of decreased cellular labeling. (C) High power magnification of peripheral areas of increased labeling. (D) High power magnification of control-treated CAM. Representative sections (10 μm) were processed for autoradiography as described in Materials and Methods and stained with toluidine blue. fb, Fibroblasts; ae, allantoic epithelium. Bars: (A) 200 μm ; (B, C, and D) 50 μm .

the density of endothelial cells below the SEC demonstrated an unexpected sharp decrease in density. As described above, it was not until 16 h that increased numbers of small blood vessels and capillaries were observed in the CAM mesenchyme. In concordance with this observation, the density of endothelial cells beneath the SEC did not begin to increase until 16 h, and it reached a peak density at 48 h, ~ 2 -fold higher than in controls. Since the SEC appeared to expand during the first 24 h of TGF β 1 treatment (compare Fig. 3, *a*, *d*, and *f*), our measurements probably underestimate the true increases in CAM endothelial cell density. Nevertheless, TGF β 1-stimulated increases in endothelial cell density were delayed relative to the multiple effects of TGF β 1 on CAM fibroblasts and allantoic epithelial cells.

Effect of TGF β 1 on Cell Proliferation

To determine the contribution of cellular proliferation in TGF β 1-stimulated density effects, ^3H -thymidine was used to label nuclei of actively proliferating cells. After 8 h of TGF β 1 (50 ng) treatment, a zone of proliferative inhibition was noted, corresponding to the area of TGF β 1-induced fibroblast and epithelial accumulation (Fig. 5 *a*). Within this central zone of proliferative inhibition (Fig. 5 *b*), the ^3H -thymidine labeling of both fibroblasts and allantoic epithelial cells was significantly diminished as compared to peripheral regions (Fig. 5 *c*) and control-treated CAMs (Fig. 5 *d*). Furthermore, note that the diminished cellular labeling in Fig. 5 *b* does not take into account the increased fibroblast and allantoic epithelial cell densities observed after 8 h of TGF β 1 treatment (e.g., Figs. 3 *b* and 4). The proliferative rates of chorionic epithelial cells and endothelial cells within the SEC could not be assessed at 8 h since these cells could not be adequately differentiated from each other.

The antiproliferative effect of TGF β 1 was sustained over 24 h (Fig. 6 *a*). At this time, the total area of inhibition was only slightly expanded as compared to at 8 h, but the border of proliferative inhibition appeared to have sharpened (compare Figs. 6 *b* and 5 *c*). Additionally, a focus of highly proliferative basal chorionic epithelial cells was usually seen just peripheral to the central region of chorionic epithelial stratification (Fig. 6 *b*).

TGF β 1-stimulated increases in endothelial cell density appeared to be mediated primarily through increased proliferation. At 24 h of TGF β 1 (50 ng) treatment, two subsets of endothelial cells were distinguishable by their labeling indices. Endothelial cells within the SEC demonstrated a very low percentage of labeled nuclei (Fig. 6, *b* and *c*). Yet, the endothelial cells beneath the SEC, within radiating capillary cords and small blood vessels, demonstrated a high percentage of labeled nuclei (Fig. 6 *c*). Note that as capillary cords approached the SEC, the nuclear labeling of endothelial cells diminished (Fig. 6 *c*). At 24 h of TGF β 1 treatment, $\sim 49\%$ of the endothelial cells within radiating capillaries and blood vessels were labeled, while in control-treated CAMs only 17% of these endothelial cells were labeled ($P < 0.001$, $n = 6$, two-tailed t test). Since at this time endothelial cells beneath the SEC reached a density ~ 2 -fold higher than in controls and a proliferative rate ~ 3 -fold higher than in controls; endothelial cell migration from more distant sites probably does not contribute significantly to the changes in endothelial cell density stimulated by TGF β 1.

Discussion

Due to the seemingly disparate *in vivo* and *in vitro* effects of TGF β on endothelial cells, we have examined the progression of cellular responses that occur during TGF β 1-induced angiogenesis with hope of gaining mechanistic insight. TGF β 1 induced gross angiogenesis when delivered to the chicken CAM. This induction was apparent within 24 h of treatment, it was dose responsive, and it was qualitatively different from the angiogenesis induced by maximal doses of bFGF. Histologic analysis revealed that TGF β 1 induced rapid, pleiotropic cellular responses. At 4 h after TGF β 1 treatment, both fibroblasts and epithelial cells accumulated in the central area of TGF β 1 delivery. This was apparently due to the migration of these cells from peripheral areas since their proliferative rates were markedly reduced in the central area at 8 and 24 h.

The rapidity of fibroblast and epithelial cell responses and their diminished magnitude with increasing distance from the methylcellulose disk suggest that TGF β 1 diffuses rapidly into the CAM and establishes a concentration gradient of biologic activity. Although it was not determined whether the CAM fibroblast and epithelial cell responses were the result of direct TGF β 1 action, the direct effects of TGF β 1 on similar cells cultured *in vitro* support this hypothesis. *In vitro* experiments have demonstrated that TGF β 1 inhibits the proliferation of most cell types (reviewed in Moses and Lyons, 1990). TGF β 1 stimulates fibroblast chemotaxis (Postlethwaite et al., 1987), morphologic alteration (Shipley et al., 1984), delayed cell cycle kinetics (Shipley et al., 1985; Sorrentino and Bandyopadhyay, 1989), and extracellular matrix production (Igotz and Massague, 1986; Fine and Goldstein, 1987). TGF β 1 has also been shown to stimulate chemotaxis of human keratinocytes (Nickloff et al., 1988). These examples of TGF β 1 effects *in vitro* are consistent with our *in vivo* observations.

Previous studies involving *in vivo* TGF β 1 administration have not separated the effects of TGF β 1 on cellular migration and proliferation (Pierce et al., 1989*a*, *b*; Roberts et al., 1986; Sprugel et al., 1987). The relative contributions of TGF β 1-stimulated cellular migration versus proliferation in achieving increased cellular density *in vivo* are unknown (Pierce et al., 1988). Our findings indicate that TGF β 1 induced CAM fibroblast and allantoic epithelial cell accumulation primarily through chemotaxis. Once these cells migrated into the central area of TGF β 1 delivery their proliferation was inhibited. These observations are consistent with *in vitro* studies which demonstrated that TGF β 1 induces cellular chemotaxis at concentrations much lower than those required to elicit other responses, including growth inhibition (Postlethwaite et al., 1987; Wahl et al., 1987). The question then arises as to the precise origin of the migrating cells and how their emigration from this location in response to TGF β 1 is compensated. Preliminary experiments suggest that the marked chemotaxis of fibroblasts and epithelial cells may be compensated by an annulus of highly proliferative cells surrounding the area of cellular accumulation. Perhaps the induction of stimulatory factors by TGF β 1, such as PDGF (Leof et al., 1986; Soma and Grotendorst, 1989), would lead to concentration-dependent proliferative signals. In distal areas, where TGF β 1 levels would be diminished, indirect stimulatory effects might then predominate over the direct inhibitory effects of TGF β 1.

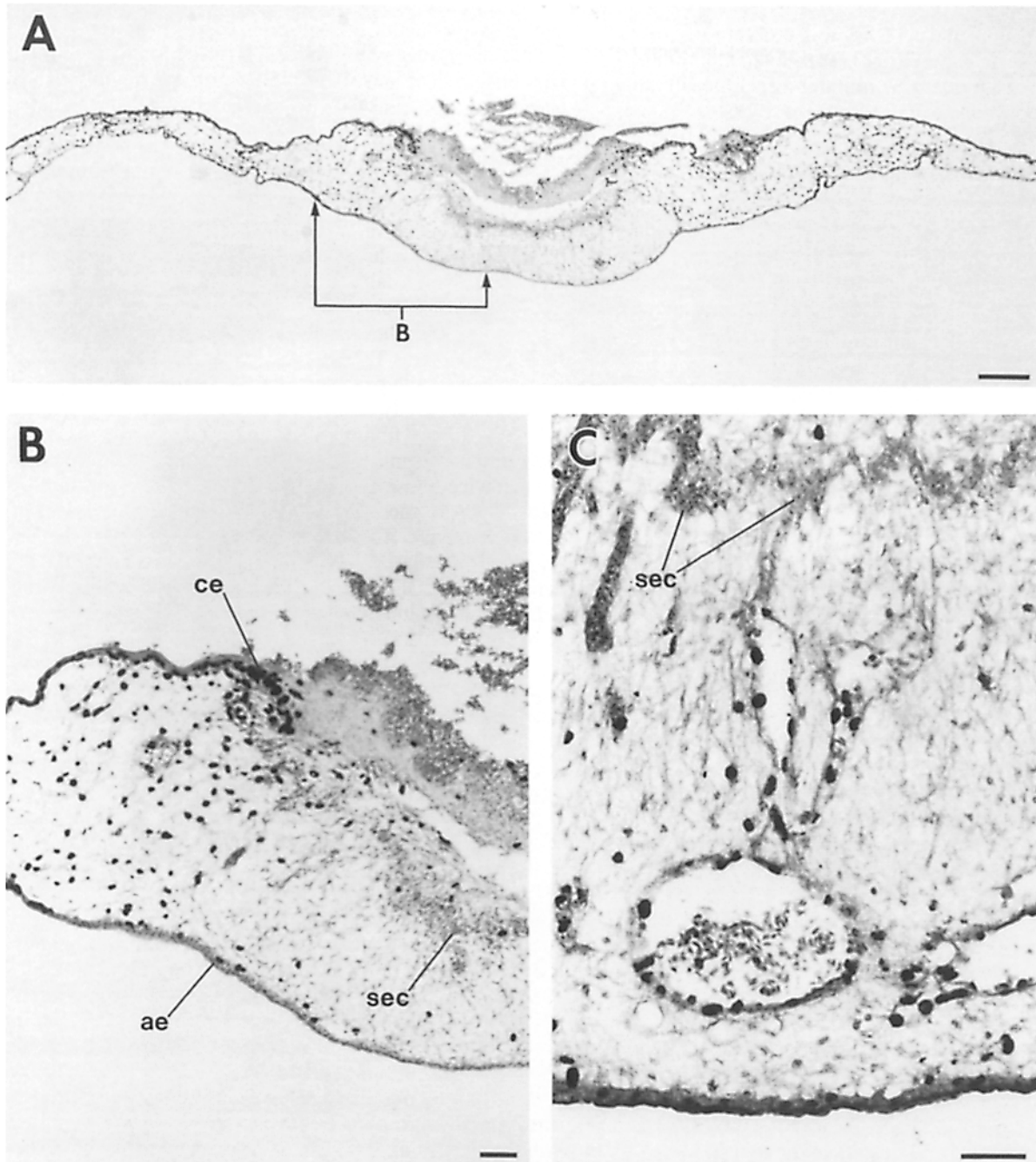


Figure 6. CAM cellular proliferation after 24 h TGF β 1 treatment as revealed by ^3H -thymidine labeling from 20 to 24 h. (A) The zone of proliferative inhibition was sustained throughout 24 h. B represents the area shown at higher magnification below. (B) High power magnification of transitional area between zone of proliferative inhibition and peripheral areas. As compared to at 8 h, the border demarcating these areas appeared to sharpen, and additionally, there was usually an adjacent focus of highly proliferative chorionic epithelial cells (ce). (C) Note that as capillary cords approached and connected with the SEC (sec) the endothelial cell labeling diminished. Representative sections (10 μm) were processed for autoradiography as described in Materials and Methods and stained with toluidine blue. ae, Allantoic epithelium. Bars: (A) 200 μm ; (B and C) 50 μm .

In contrast to the rapid effects of TGF β 1 on CAM fibroblasts and epithelial cells, the effects of TGF β 1 on endothelial cells were delayed. Initially, the endothelial cell density declined, perhaps as a result of the rapid accumulation of fibroblast and epithelial cells. Increases in endothelial cell density were first observed 16 h after TGF β 1 treatment, whereas fibroblast and epithelial cell densities increased by

4 h. Capillary cords developed in a radial pattern relative to the point of TGF β 1 delivery, and they were often incomplete in their connection to the SEC. These observations suggest that radial capillary cords originated from larger blood vessels and extended primarily through growth towards the SEC. It has been demonstrated that the growth and extension of capillaries takes place by the combined migration of en-

endothelial cells at the leading tip and proliferation of endothelial cells behind the tip (Ausprunk and Folkman, 1977). This spatial pattern of endothelial cell proliferation was also observed during TGF β 1-induced angiogenesis since the percentage of ^3H -thymidine-labeled endothelial cells within radiating capillaries and blood vessels was higher in distal areas as compared to proximal areas. As capillary cords approached and incorporated into the SEC, the proliferation of endothelial cells within these vessels was inhibited.

The observation that endothelial cells in central areas changed from a highly proliferative state, lining angiogenic capillary cords, to a quiescent state, lining the SEC, is consistent with in vitro data demonstrating that TGF β 1 is directly inhibitory to the processes required to initiate angiogenesis (i.e., invasion, migration, proliferation) and directly stimulatory to the processes required to resolve angiogenesis (i.e., capillary morphogenesis). The increased endothelial cell proliferation in peripheral regions is difficult to explain however. At low concentrations, TGF β 1 could directly stimulate endothelial cell proliferation, but concentrations as low as 10 pg/ml are reportedly inhibitory to proliferation in vitro (Baird and Durkin, 1986; Frater-Schroder et al., 1986). A mechanism of indirect stimulation, via production of an autocrine stimulatory factor by endothelial cells is also possible, and in fact TGF β 1 has been shown to induce increased bFGF production in bovine corneal endothelial cells (Plouet and Gospodarowicz, 1989). Finally, TGF β 1 could induce endothelial cell proliferation at low concentrations by stimulating production of a paracrine stimulatory factor from an intermediate cell type. Since angiogenic capillary cords were observed to grow through regions of previous fibroblast accumulation and extracellular matrix deposition, it is possible that fibroblasts contribute an active role in TGF β 1-induced angiogenesis.

Although macrophages clearly have an important role in wound healing (Leibovich and Ross, 1975; Danon et al., 1989) and its associated angiogenesis (Polverini et al., 1977; Koch et al., 1986), we did not find an association between endothelial cell growth and the presence of macrophages or PMNs. Previous work has suggested that wound-healing angiogenesis can occur without inflammation (Eliason, 1978; Sholley et al., 1978). It has been hypothesized that wound-healing angiogenesis is initiated without inflammation, but amplified maximally through further secretion of angiogenic substances by macrophages (Eliason, 1978). This hypothesis is in agreement with our observations of TGF β 1-induced angiogenesis, which appeared to be initiated at the microvascular level in an inflammation-independent manner and remodeled into larger vessels in association with mononuclear inflammation.

Several recent findings suggest the broader significance of TGF β 1-induced cellular accumulation and angiogenesis. Both TGF β 1 and TGF β 2 are developmentally expressed in tissues where angiogenesis occurs within organizing mesenchyme. In embryonic mouse limbs, TGF β 1 immunohistochemical staining is most profound in the developing mesenchyme surrounding the marginal blood sinuses (Heine et al., 1987). In embryonic mouse skin, both TGF β 1 immunohistochemical staining (Heine et al., 1987) and TGF β 2 mRNA (Pelton et al., 1989) are localized in the subepidermal mesenchyme during periods of increased capillary growth and extracellular matrix organization. The capacity of tumors to

induce the production of angiogenic factors in normal tissues has also been suggested (Folkman, 1984). Many tumor cell lines that produce and secrete TGF β 1 are also highly desmoplastic and angiogenic in vivo (Bergh, 1988; Beauchamp et al., 1990). Thus, the capacity of TGF β 1 to initiate a program of cellular responses which ultimately results in connective tissue remodeling and angiogenesis may be a conserved process utilized in embryogenesis, recapitulated in wound healing, and exploited in tumor progression.

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