Transforming Growth Factor β1–induced Changes in Cell Migration, Proliferation, and Angiogenesis in the Chicken Chorioallantoic Membrane

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Abstract. Application of TGFβ1 (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 TGFβ1 were qualitatively different than those induced by maximal doses of basic FGF (bFGF) (500 ng). While TGFβ1 induced the formation of large blood vessels by 72 h, bFGF induced primarily small blood vessels. Histologic analysis revealed that TGFβ1 stimulated pleiotropic cellular responses in the CAM. Increases in fibroblast and epithelial cell density in the area of TGFβ1 delivery were observed as early as 4 h after TGFβ1 treatment. By 8 h, these cell types also demonstrated altered morphology and marked inhibition of proliferation as evidenced by 3H-thymidine labeling. Thus, the TGFβ1-stimulated accumulation of these cell types was the result of cellular chemotaxis from peripheral areas into the area of TGFβ1 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 TGFβ1 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, TGFβ1 initiates a sequence of cellular responses that results in growth inhibition, cellular accumulation through migration, and microvascular angiogenesis.

Transforming growth factor beta (TGFβ) 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, TGFβ was identified in many normal tissues (Proper et al., 1982; Roberts et al., 1982), additional TGFβ biologic activities were discovered (reviewed in Moses et al., 1988), and several growth factors closely related to TGFβ were identified (TGFβ1, TGFβ2, and TGFβ3; reviewed in Lyons and Moses, 1990). Our concepts of the roles of TGFβ in vivo have therefore evolved.

Our best understanding of TGFβ function in vivo is in wound healing. TGFβ is stored in platelets (Childs et al., 1982) and released from platelets during degranulation (Assoian and Sporn, 1986). TGFβ 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 TGFβ 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 TGFβ1, the prototypic TGFβ, yields a wound healing response. When TGFβ1 is injected subcutaneously into newborn mice a granulation tissue response characterized by inflammation, fibrosis, and angiogenesis is observed (Roberts et al., 1986). TGFβ1 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 TGFβ has not yet been shown to impair normal wound healing, TGFβ is most likely a natural mediator of wound healing.

To understand the mechanisms by which TGFβ might function in wound healing and other processes, the effects of TGFβ have been studied on isolated cells in vitro. These...
Figure 1. CAM vascular reactions to TGFβ1 and bFGF. A control methylcellulose disk (A) and methylcellulose disks impregnated with either 50 ng TGFβ1 (B and D) or 500 ng bFGF (C) were placed onto the CAM for 72 h (A–C) or 24 h (D). TGFβ1 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), TGFβ1 consistently induced the formation of much larger blood vessels. This was the result of vascular remodeling since much smaller blood vessels were induced by TGFβ1 after only 24 h (arrowhead, D). Bars, 1 mm.

In vitro endothelial cell assays that measure endothelial cell protease production, invasion, migration, or proliferation model early events in angiogenesis (Folkman, 1985). TGFβ1 is inhibitory in these assays. TGFβ1 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., 1988).

Figure 2. Dose-responsive effect of TGFβ1 on mean angiogenic scores. Control methylcellulose disks (Cntl) and methylcellulose disks impregnated with either 10, 50, or 100 ng of TGFβ1 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 ± SEM, n > 16. (*) P < 0.001 as compared to control.
TGF/β 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 inhibits PMA-induced endothelial cell invasion and subsequent capillary tube formation (Müller et al., 1987). Taken collectively, the direct effects of TGF/β on endothelial cells in vitro might suggest that TGF/β is a general repressor of angiogenesis. Yet, administration of TGF/β 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-induced angiogenesis.

Materials and Methods

Embryos and Reagents

Fertilized white leghorn chicken eggs were obtained from Truslow Farms (Chestertown, MD). Porcine TGF/β1 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/β1 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-μm 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 μCi of [3H]-thymidine, 1.0 μCi/ml (980 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-μm 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/β1-treated CAM tissue samples were examined for total endothelial cell numbers and total [3H]-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 μm 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 TGFβ1

To examine the effects of TGFβ1 on the CAM vasculature TGFβ1-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. 1a). However, application of a TGFβ1-impregnated methylcellulose disk (50 ng) for the same period of time resulted in a marked angiogenic effect (Fig. 1b). TGFβ1 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 TGFβ1 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 TGFβ1. A maximally effective dose of bFGF (500 ng or higher) induced angiogenic effects that were qualitatively different than the effects induced by TGFβ1. After 72 h, when the vascular effects were greatest, bFGF induced the growth of very small blood vessels (Fig. 1c), as compared to the larger vessels induced by TGFβ1 (Fig. 1b). Because the mechanism of TGFβ1-induced angiogenesis could be through the production of bFGF, we examined TGFβ1-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 TGFβ1 administration (Fig. 1d). Although small
Figure 3.
Figure 3. Histologic effects induced by TGFβ1 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 TGFβ1 (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 TGFβ1 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 TGFβ1 delivery. (I) At 72 h, mononuclear infiltration was evident (open arrowheads) in a perivascular distribution (bv, blood vessel). Representative sections (10 μm) were stained with hematoxylin and eosin. Bars: (A-D, F-I) 50 μm; (E) 20 μm.

Microscopic Effects Induced by TGFβ1

The progression of TGFβ1-induced angiogenesis was then examined histologically. Serial cross sections were made through samples of CAMs treated with TGFβ1 (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 TGFβ1-impregnated methylcellulose disks (50 ng) to the CAM resulted in alterations in fibroblast, epithelial, and endothelial cell morphology. After an 8-h exposure to TGFβ1, 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) infiltration 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 TGFβ1-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 ~16 h after TGFβ1 treatment. Capillaries within the SEC appeared to be more numerous and more densely packed at this
Over the course of 24 h the area of TGFß1-induced effects enlarged and earlier responses were amplified. 24-h TGFß1-treated CAMs were ~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 TGFß1 delivery (Fig. 3 f). This radial pattern was reiterated in the developing capillary cords which extended from larger blood vessels towards the point of TGFß1 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 TGFß1-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 TGFß1 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 TGFß1 on Cell Density

TGFß1 has been shown to affect both cellular chemotaxis and proliferation. To separate the effects of TGFß1 on these two processes in the CAM, the changes in cellular density and 3H-thymidine incorporation after TGFß1 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 TGFß1 (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 ~8 h after TGFß1 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 TGFß1 effect were counted. The density of this population of fibroblasts increased ~3.5-fold during the first 4 h of TGFß1 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 ~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 μm 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 TGFβ1 treatment as revealed by [3H]-thymidine labeling from 4 to 8 h. (A) CAMs treated with TGFβ1 (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 TGFβ1 treatment (compare Figs. 3, a, d, and f), our measurements probably underestimate the true increases in CAM endothelial cell density. Nevertheless, TGFβ1-stimulated increases in endothelial cell density were delayed relative to the multiple effects of TGFβ1 on CAM fibroblasts and allantoic epithelial cells.

**Effect of TGFβ1 on Cell Proliferation**

To determine the contribution of cellular proliferation in TGFβ1-stimulated density effects, 3H-thymidine was used to label nuclei of actively proliferating cells. After 8 h of TGFβ1 (50 ng) treatment, a zone of proliferative inhibition was noted, corresponding to the area of TGFβ1-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 TGFβ1 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 TGFβ1 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).

TGFβ1-stimulated increases in endothelial cell density appeared to be mediated primarily through increased proliferation. At 24 h of TGFβ1 (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 TGFβ1 treatment, ~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 TGFβ1.

**Discussion**

Due to the seemingly disparate in vivo and in vitro effects of TGFβ1 on endothelial cells, we have examined the progression of cellular responses that occur during TGFβ1-induced angiogenesis with hope of gaining mechanistic insight. TGFβ1 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 TGFβ1 induced rapid, pleiotropic cellular responses. At 4 h after TGFβ1 treatment, both fibroblasts and epithelial cells accumulated in the central area of TGFβ1 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 TGFβ1 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 TGFβ1 action, the direct effects of TGFβ1 on similar cells cultured in vitro support this hypothesis. In vitro experiments have demonstrated that TGFβ1 inhibits the proliferation of most cell types (reviewed in Moses and Lyons, 1990). TGFβ1 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 (Ignotz and Massague, 1986; Fine and Goldstein, 1987). TGFβ1 has also been shown to stimulate chemotaxis of human keratinocytes (Nickoloff et al., 1988). These examples of TGFβ1 effects in vitro are consistent with our in vivo observations.

Previous studies involving in vivo TGFβ1 administration have not separated the effects of TGFβ1 on cellular migration and proliferation (Pierce et al., 1989a, b; Roberts et al., 1986; Sprugel et al., 1987). The relative contributions of TGFβ1-stimulated cellular migration versus proliferation in achieving increased cellular density in vivo are unknown (Pierce et al., 1988). Our findings indicate that TGFβ1 induced CAM fibroblast and allantoic epithelial cell accumulation primarily through chemotaxis. Once these cells migrated into the central area of TGFβ1 delivery their proliferation was inhibited. These observations are consistent with in vitro studies which demonstrated that TGFβ1 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 TGFβ1 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 TGFβ1, such as PDGF (Leof et al., 1986; Soma and Grotendorst, 1989), would lead to concentration-dependent proliferative signals. In distal areas, where TGFβ1 levels would be diminished, indirect stimulatory effects might then predominate over the direct inhibitory effects of TGFβ1.
Figure 6. CAM cellular proliferation after 24 h TGF/β1 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/β1 on CAM fibroblasts and epithelial cells, the effects of TGF/β1 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/β1 treatment, whereas fibroblast and epithelial cell densities increased by 4 h. Capillary cords developed in a radial pattern relative to the point of TGF/β1 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 TGFβ1-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 TGFβ1 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, TGFβ1 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 TGFβ1 has been shown to induce increased bFGF production in bovine corneal endothelial cells (Plouet and Gospodarowicz, 1989). Finally, TGFβ1 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 TGFβ1-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 TGFβ1-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 TGFβ1-induced cellular accumulation and angiogenesis. Both TGFβ1 and TGFβ2 are developmentally expressed in tissues where angiogenesis occurs within organizing mesenchyme. In embryonic mouse limbs, TGFβ1 immunohistochemical staining is most profound in the developing mesenchyme surrounding the marginal blood sinuses (Heine et al., 1987). In embryonic mouse skin, both TGFβ1 immunohistochemical staining (Heine et al., 1987) and TGFβ2 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 TGFβ1 are also highly desmoplastic and angiogenic in vivo (Bergh, 1988; Beauchamp et al., 1990). Thus, the capacity of TGFβ1 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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