with its final position within the retina (*5*). Thus, postmitotic cells that migrate away from the "outer limiting membrane" and toward the inner retina develop as neurons, whereas those that fail to migrate develop as photoreceptors. Cell migration is extensive during the ED-6 to ED-8 period. Therefore, developmental stage–dependent changes in the frequency of differentiated phenotypes observed in vitro are consistent with a model of retinal cell differentiation (Fig. 3D) in which (i) precursor cells remain uncommitted for some time after terminal mitosis, (ii) they differentiate as neurons only after relocating to the inner retina where they are exposed to position-dependent regulatory signals, and (iii) precursor cells that fail to relocate to "neuron-inducing" retinal regions follow a constitutive or "default" pathway and differentiate as photoreceptors. In vivo, this constitutive developmental pathway is followed by those postmitotic cells that remain attached to the outer limiting membrane and have their cell body located in the outer nuclear layer. A similar fate would be followed in vitro by those postmitotic cells that are dissociated from the retina before their migration.

Using retroviral markers to trace cell lineages in the rat retina, Turner and Cepko (*1*) observed that rods, bipolar cells, and amacrine cells have common lineage until late in development, suggesting that determination of cell type may occur at or after the final mitosis of precursor cells. Wetts and Fraser injected fluorescent dextrans into cells of the immature frog optic vesicle and obtained similar results (*2*). Our in vitro observations are consistent with and complementary to the conclusions derived from lineage analysis. However, further studies are needed to determine whether the cells that differentiate as photoreceptors when isolated from the embryo at ED-6 are the same cells as those that differentiate as neurons when isolated at ED-8. This uncertainty is due to the presence, in all of the cultures studied, of some cells that fail to express any differentiated phenotype and remain as process-free, round cells. Our culture technique offers an experimental system for investigating this question and ascertaining whether the fate of individual postmitotic precursor cells changes in response to different microenvironmental conditions.

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8. Cultures of dissociated chick retinal neurons and photoreceptors, free of contamination by connective tissue, glial, vascular endothelial, and pigment epithelial cells, were established as described (*3*). Neural retinas were dissected from White Leghorn chick embryos, briefly trypsinized and dissociated by trituration. The dissociated cells were suspended in medium 199 supplemented with 10% heat-inactivated fetal bovine serum, linoleic acid–albumin (110 μg/ml), and penicillin (100 U/ml) and seeded in polyornithine-coated tissue culture dishes. Glial cells do not develop under these culture conditions (*3*).
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Interleukin-1 Mitogenic Activity for Fibroblasts and Smooth Muscle Cells Is Due to PDGF-AA

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Both interleukin-1 (IL-1) and platelet-derived growth factor (PDGF) induce proliferation of cultured fibroblasts and smooth muscle cells. These polypeptide mediators are released by activated macrophages and other cell types in response to injury and are thought to have a role in tissue remodeling and a number of pathologic processes. Analysis of the kinetics of [^{3}H]thymidine incorporation by cultured fibroblasts demonstrated that the response to IL-1 is delayed approximately 8 hours relative to their response to PDGF. IL-1 transiently stimulated expression of the PDGF A-chain gene, with maximum induction after approximately 2 hours. Subsequent synthesis and release of PDGF activity into the medium was detected as early as 4 hours after IL-1 stimulation, and downregulation of the binding site for the PDGF-AA isoform of PDGF followed PDGF-AA secretion. Antibodies to PDGF completely block the mitogenic response to IL-1. Therefore, the mitogenic activity of IL-1 for fibroblasts and smooth muscle cells appears to be indirect and mediated by induction of the PDGF A-chain gene.

THE INTERLEUKIN-1 MOLECULES (IL-1α and IL-1β) are multipotent inflammatory mediators that are produced by activated macrophages and by epidermal, lymphoid, and vascular cells. They have the capacity to affect mesenchymal destructive and reparative processes during tissue remodeling (*1*). The effects on cultured connective tissue cells include proliferation of fibroblasts (*2*), Balb/3T3 cells (*3, 4*), and smooth muscle cells (*5*), as well as stimulation of collagenase and prostaglandin E_2 production (*6*). Confluent fibroblast monolayers express 5,000 to 15,000 IL-1 receptors per cell that bind both IL-1α and IL-1β (*3, 7*). However, in comparison with platelet-derived growth factor (PDGF), IL-1 does not induce early changes in specific cellular proteins in Balb/3T3, and IL-1 produces relatively small changes in DNA synthesis (*4*). In human fibroblasts, IL-1 receptor–mediated ligand internalization is slow (*7*) and the kinetics of IL-1–induced increases in mRNA for c-*fos* and c-*myc* differs from the kinetics of other growth factor–stimulated increases (*8*). PDGF and IL-1 are both secreted by activated macrophages and are potential mediators of fibroblast proliferation (*1, 9*). In this study, we compared the relative mitogenic potencies of the three isoforms of PDGF (PDGF-AA, PDGF-BB, and PDGF-AB) and of IL-1 for human dermal fibroblasts and arterial smooth muscle cells. Our data demonstrate that IL-1–induced proliferation is not due to direct mitogenic stimulation by IL-1 but is mediated by induction and release of PDGF-AA by fibroblasts or smooth muscle cells in an autocrine or paracrine manner, or both.

Initial studies comparing the relative mitogenic activities of IL-1 and PDGF, as measured by [^{3}H]thymidine incorporation at 18 to 20 hours after mitogen addition, demonstrated little or no response to IL-1. Consequently, the kinetics of thymidine incorporation were examined over 48 hours at 8- to 12-hour intervals. The first increase in [^{3}H]thymidine incorporation in response to PDGF-AA, PDGF-BB, and PDGF-AB was seen at 16 hours and was maximal at 24

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hours (Fig. 1). In contrast, with either IL-1α or IL-1β, an increase in [^{3}H]thymidine incorporation was not detected until 24 hours and was maximal at 36 hours. Since the kinetics of [^{3}H]thymidine incorporation for serum-stimulated fibroblasts are similar to those with PDGF, the delay observed with IL-1 is consistent with the interpretation that IL-1 stimulates the expression and translation of a biologically active molecule that then induces the mitogenic stimulation. Similar delayed kinetics were the basis for experiments demonstrating that in AKR-2B cells, transforming growth factor–β indirectly stimulates growth, at least partially, by induction of the PDGF B chain and release of PDGF activity (*10*). In earlier studies of IL-1 mitogenic activity for fibroblasts and smooth muscle cells, [^{3}H]thymidine incorporation was measured 2 to 4 days after IL-1 addition (*2, 5*).

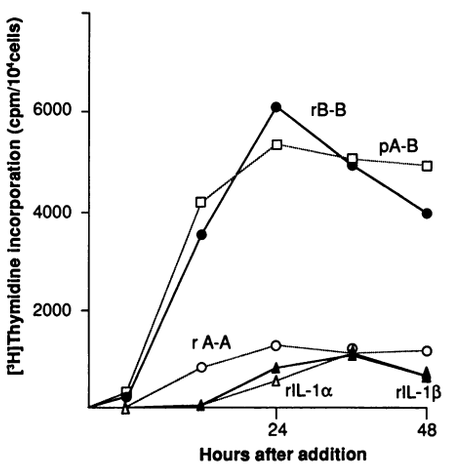

Fig. 1. Delayed kinetics of IL-1-induced mitogenic stimulation of fibroblasts. Human dermal fibroblasts were plated in 24-well culture dishes (2.5×10^4 per well) in 1 ml of 1% human plasma-derived serum (PDS) (*21*) or 1% calf serum. Four to five days after plating, recombinant IL-1α (△) or IL-1β (▲) (0.25 ng/ml) (*22*), or purified yeast-derived PDGF-AA (110 amino acid endothelial form) (*23*) (○) or PDGF-BB (109 amino acids) (●) (1 ng/ml) (*24*), or platelet-derived PDGF-AB (□) were added to the wells at time 0 and cultures were incubated at 37°C. Platelet-derived PDGF-AB was partially purified from platelet-rich plasma as previously described (*21*) and then affinity-purified by passage over Sepharose-bound monoclonal antibody 120.1.2.1.1 to remove PDGF-BB and then over a second affinity column, Sepharose-bound monoclonal antibody 121.6.1.1.1, which binds the PDGF B chain of PDGF-AB. At the times indicated, the medium was removed and replaced with [^{3}H]thymidine (2 μCi/ml) and, after a 2-hour pulse at 37°C, incorporation of [^{3}H]thymidine into trichloroacetic acid–precipitable material was determined as previously described (*21*). Background [^{3}H]thymidine incorporation in wells receiving only diluent were subtracted (300 cpm per 10^4 cells) and counts per minute per 10^4 cells at each time point have been plotted. Each point is the mean of triplicate wells, and standard errors were always less than 5% of the mean.

We evaluated dose-response curves for PDGF-AA, PDGF-BB, and PDGF-AB and for IL-1α and IL-1β at different times after mitogen addition. Doses giving maximal [^{3}H]thymidine incorporation were used in the kinetic analysis (Fig. 1) (*11*). Maximal [^{3}H]thymidine incorporation, detected at 24 to 26 hours in response to PDGF-BB, was approximately six times that seen in response to PDGF-AA (Fig. 1). The human dermal fibroblasts used in this study have approximately 20 times as many PDGF-BB binding sites as PDGF-AA binding sites (*12, 13*). The extent of [^{3}H]thymidine incorporation detected at 36 to 38 hours after the addition of IL-1 is identical to the maximal response detected at 24 to 26 hours with PDGF-AA (Fig. 1).

Binding of any of the three isoforms of PDGF to cells at 37°C leads to depletion of the specific PDGF binding sites through receptor internalization (*12*). To determine whether binding sites for PDGF-AA or PDGF-BB were modulated in response to IL-1, we incubated human fibroblasts at 37°C with or without IL-1α (0.25 ng/ml), and evaluated the relative expression of these binding sites at 4°C. At 48 and 72 hours after IL-1 addition, only a small decrease in binding sites specific for PDGF-BB was detected by measuring binding of the monoclonal antibody PR7212 (Fig. 2) (*12, 13*). However, PDGF-AA binding sites, evaluated by binding of ^{125}I-labeled PDGF-AA, decreased to 10% of the untreated level at 36 hours, were undetectable at 48 hours, and returned to control values by 72 hours in IL-1–treated cells. This is not due to a

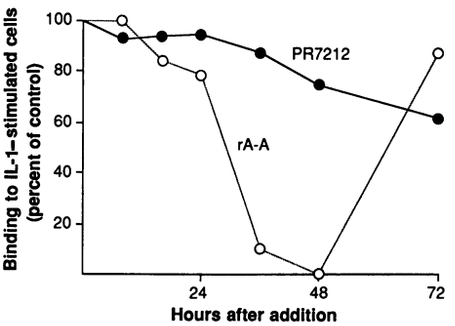

Fig. 2. IL-1 transiently downregulates PDGF-AA binding. Human dermal fibroblasts were plated as described in Fig. 1. At time 0, half of each tray received diluent and the other half received IL-1α (0.25 ng/ml). At the indicated times, the cells were cooled to 4°C and incubated for 3 hours at 4°C on a shaking platform with ^{125}I-labeled PDGF-AA (○) or ^{125}I-labeled antibody PR7212 (●) both in the presence and absence of 100-fold excess (100 ng/ml and 10 μg/ml, respectively) of unlabeled competitor. The cells were washed, harvested with Triton X-100 solubilization buffer, and total cell-associated counts per minute were determined as previously described (*24*). The data with IL-1–treated cells was plotted as the mean percentage of diluent-treated cells for determinations made from triplicate wells of a representative experiment, PDGF-AA and antibody PR7212 were labeled with ^{125}I with the use of iodobeads (Pierce Chemical Company) as described by the manufacturer, giving specific activities of approximately 6×10^7 cpm/μg and 2×10^7 cpm/μg, respectively. Nonspecific binding, determined by the addition of 100 times excess of unlabeled ligand, was 40% of the total binding.

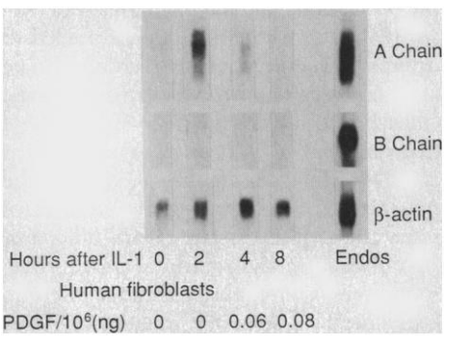

Fig. 3. IL-1 transiently induces expression of PDGF A chain but not B chain, and PDGF activity is detectable in the media. Confluent plates (150-cm diameter) of human dermal fibroblasts (approximately 3×10^6 cells per plate) in medium containing 1% human PDS (*21*) were treated with IL-1α (0.25 ng/ml). At the indicated times, the media were collected and cells were trypsinized, counted, and pelleted for RNA isolation. The level of PDGF was determined by PDGF radioreceptor assay with ^{125}I-labeled PDGF-AB (*25*). Media from matched maxi plates that received diluent only were harvested at time 0, 24, and 36 hours to evaluate basal levels of secretion of PDGF activity, which varied from <0.02 ng per 10^6 cells per 24 hours to 0.05 ng per 10^6 cells per 24 hours. RNA was isolated from the pelleted cells by direct lysis of cells (*26*). Poly(A)$^+$ RNA (4 μg) from each time point or 15 μg of total RNA (*27*), isolated from human umbilical vein endothelial cells (Endos), were separated in a 1% agarose gel containing formaldehyde, and the RNA was transferred electrophoretically to 0.45-μm Nytran filters (Schleicher & Schuell). Filters were prehybridized for 4 hours and hybridized overnight at 42°C (B chain and β-actin) or 50°C (A chain) with 5×10^6 cpm or 10×10^6 cpm per blot, respectively. Hybridization buffer contained 0.25*M* Na_2HPO_4, 0.25*M* NaCl, 1m*M* EDTA, 47% formamide, 7% SDS, 10% polyethylene glycol [PEG (6000)], and double-stranded salmon sperm DNA (250 μg/ml). We labeled the cDNA probes with [^{32}P]deoxycytidine 5′-triphosphate, using random-primed DNA labeling (*28*) (specific activities of 1 to 2×10^9 cpm/μg): the PDGF A chain (clone D_1, 1.3 kb) (*29*); PDGF B chain (704-bp Bam HI fragment) (*23*); and β-actin (1.33-kb Pst I fragment) (*30*). After hybridization, the membranes were washed with 0.2× saline sodium citrate (SSC), 0.1% SDS (*p*H 7.2) at 50°C (A chain and β-actin) or with 0.1× SSC, 0.1% SDS (*p*H 7.2) at 60°C (B chain). Exposure of the blots to Kodak XAR-5 x-ray film was performed at −70°C with a Cronex intensifier screen for 19 hours (A chain), 7 days (B chain), and 6 hours (β-actin).

change in the affinity of PDGF-AA binding, but rather to a decrease in PDGF-AA receptor number as determined by saturation binding studies. Similar experiments showed a threefold increase in IL-1 receptor level, measured by ^{125}I-labeled IL-1β saturation binding, after addition of PDGF. However, parallel measurements, made by flow microfluorimetry, of the fraction of cycling cells suggested that this increase was a reflection of the general mitogenic effect of PDGF and not, as suggested recently (*14*), a specific regulatory effect on IL-1 receptor expression.

Specific downregulation of the PDGF-AA binding sites and the similarity in the maximal levels of [^{3}H]thymidine incorporation in response to IL-1 and PDGF-AA suggest that PDGF-AA is expressed and secreted in response to IL-1. This interpretation is also consistent with earlier observations that the A chain of PDGF is transiently induced when fibroblasts are stimulated with epidermal growth factor and PDGF (*15*). Cellular levels of PDGF-A and PDGF-B mRNA transcripts and PDGF activity in conditioned media were evaluated at various time points after IL-1 addition. PDGF A-chain mRNA is transiently induced at 2 hours and falls to undetectable levels by 8 hours (Fig. 3). In further experiments in which PDGF-A mRNA levels were examined at 8-hour intervals up to 48 hours after IL-1 addition, a second transient increase was observed at 16 hours. PDGF activity was detected in the culture medium as early as 4 hours after IL-1 addition (Fig. 3), and continued to increase to 48 hours. No PDGF-B mRNA was detected at any of the time points examined. Monoclonal antibodies were used to evaluate the isoform responsible for the PDGF activity detected in conditioned medium. The PDGF activity from IL-1–treated fibroblasts was completely neutralized by a monoclonal antibody to PDGF-AA and PDGF-AB but was unaffected by a monoclonal antibody specific for PDGF-BB and PDGF-AB. Both the isoform-specific monoclonal antibody neutralization of the conditioned medium and the specific downregulation of the PDGF-AA binding sites are consistent with the release of PDGF-AA by IL-1–treated cells.

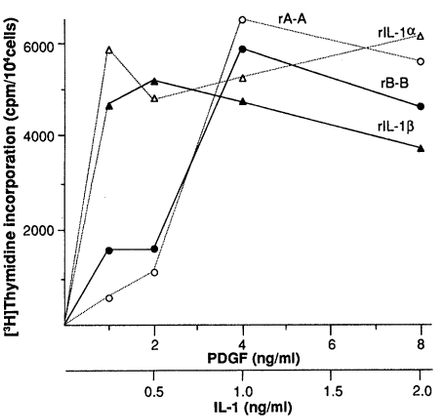

Fig. 4. The maximal mitogenic activity induced by IL-1 is comparable to PDGF-AA–induced mitogenic activity on smooth muscle cells. Newborn human thoracic aorta smooth muscle cells, isolated as previously described (*31*), were plated at 8×10^4 per well in 5% fetal calf serum in 24-well culture dishes. Four days later, the cells were changed to 1% human PDS and the experiment began 1 day later. Cell density on the day of initiation of the experiment was 9.6×10^4 cells per square centimeter. Different doses of IL-1α (△) or IL-1β (▲) or PDGF-AA (○) or PDGF-BB (●) were added at time 0. Indomethacin (1 μg/ml) was added to all wells receiving IL-1α and IL-1β as previously described for smooth muscle cells (*5*). At 42 to 44 hours, the cultures were pulsed with [^{3}H]thymidine, and incorporation was determined as described in Fig. 1. Background [^{3}H]thymidine incorporation in wells receiving diluent only were subtracted (4936 cpm per 10^4 cells), and counts per minute per 10^4 cells at each dose have been plotted. Each point is the mean of triplicate wells, and standard errors were always less than 10% of the mean.

To determine whether the maximal response induced by IL-1 would mimic the maximal response induced by PDGF-AA in other cells with different relative PDGF-AA and PDGF-BB binding sites, we examined IL-1 stimulation of human aortic smooth muscle cells. On confluent human smooth muscle cells, the numbers of PDGF-AA and PDGF-BB binding sites are approximately equal and the maximal mitogenic activity of PDGF-AA is equivalent to that seen with PDGF-BB (Fig. 4). The maximal mitogenic activity induced at 42 to 44 hours by IL-1 in smooth muscle cells is also equivalent to that induced by PDGF-BB and PDGF-AA (Fig. 4). Therefore, in both fibroblasts and smooth muscle cells, the maximal IL-1–induced mitogenic response is comparable to that observed with PDGF-AA.

These data demonstrate that the mitogenic activity for fibroblasts and smooth muscle cells, previously ascribed to IL-1, is principally, if not totally, due to induction of PDGF A-chain gene expression and subsequent synthesis and secretion of PDGF-AA. The secretion of PDGF-AA induces downregulation of the PDGF-AA binding sites and is consistent with the delayed induction of [^{3}H]thymidine incorporation in response to IL-1. As demonstrated for PDGF-AA, the maximal mitogenic activity relative to PDGF-BB is dependent on the relative number of PDGF-AA and PDGF-BB binding sites. It is unclear whether this proportionality reflects signaling differences between the PDGF receptor complexes or simply shows that a larger number of binding sites will by mass action achieve a steady state more readily (*16*). However, the constant relation between the maximal mitogenic signal induced by IL-1 and that induced by PDGF-AA in two cell types with different relative numbers of PDGF-AA and PDGF-BB binding sites supports the concept that PDGF-AA is the principal mitogen induced by IL-1.

Attempts to use exogenous addition of antibodies to PDGF in vitro to determine the role of PDGF in proposed autocrine systems have met with limited success (*17*). Preliminary studies in our laboratory to inhibit IL-1–stimulated mitogenesis with an antibody to PDGF at concentrations five times greater than is required to neutralize exogenously added PDGF-AA showed partial inhibition when more than one addition of antibody was made during the 42-hour incubation. However, a 25-fold excess (20 mg/ml) of an immunoglobulin G (IgG) specific for PDGF [at 0.8 mg/ml, the IgG completely inhibits exogenously added PDGF-AA (1 ng/ml)] completely inhibited the mitogenic response induced by IL-1α or IL-1β (0.25 ng/ml) in both human fibroblasts and smooth muscle cells.

Our finding that the mitogenic action of IL-1α and IL-1β on fibroblasts and smooth muscle cells is indirect and mediated by induction of PDGF A-chain synthesis and secretion is consistent with the mode of action of IL-1 in several other systems (*1*). For example, the lymphocyte-activating factor activity of IL-1 for T lymphocytes is likely mediated by induction of IL-2 and IL-6 synthesis and secretion (*18*). This pattern of activities places IL-1α and IL-1β in a group of peptide mediators whose principal action is upregulation of cellular metabolism and increased expression of several genes coding for biologically active molecules. Transforming growth factor–β and tumor necrosis factor–α have similar patterns of activity (*19, 20*). Our findings also reinforce the notion that the PDGF family, along with the epidermal growth factor and fibroblast growth factor families, are the primary growth factors responsible for regulating the proliferation of connective tissue cells.

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26. Briefly, the cells were lysed in 1 ml of 10 m*M* tris, *p*H 7.4, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, 1% SDS, 0.5% β-mercaptoethanol, 10 m*M* vanadylribonucleotide complex, and 350 μg of proteinase K per milliliter, and heated to 42°C for 15 min. Each sample was then sonicated three times for 30 s each and then heated to 42°C for another 15 min. EDTA was added to give a final concentration of 10 m*M* and incubated another 5 min at 42°C, followed by addition of LiCl to give a concentration of 0.5*M*, and then the samples were cooled to room temperature. The extracted RNA was passed over oligo(dT) cellulose [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)] to obtain poly(A)$^+$ RNA. RNA was estimated by an absorbance at 260 nm.
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Secretion of Activin by Interstitial Cells in the Testis

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Activin, a dimer formed by the β subunits of inhibin, has an effect that is opposite to that of inhibin in a number of biological systems. Which cell types secrete activin in vivo is not known. TM3 cells, a Leydig-derived cell line, contained messenger RNAs that hybridized with human β_A and β_B complementary DNA probes and were similar in size to the porcine messenger RNA for the β subunits of inhibin. No hybridization to the inhibin α subunit was detectable in the TM3 cells. Conditioned medium from TM3 cells and from primary cultures of rat and porcine interstitial cells stimulated the release of follicle-stimulating hormone in a pituitary cell culture assay. It is likely that, in the testis, the Leydig cells secrete activin and the Sertoli cells produce inhibin, or a combination of both.

INHIBIN IS A POLYPEPTIDE CAPABLE OF suppressing follicle-stimulating hormone (FSH) release from the pituitary without affecting the release of leutinizing hormone (LH) (*1*). The isolation, cloning, and sequencing of the inhibin cDNA showed that inhibin is a heterodimer composed of an α and one of two possible β chains (β_A or β_B) (*2–5*). Inhibin activity has been isolated from both the ovary and the testis (*1, 6, 7*). During the purification of inhibin from porcine follicular fluid, proteins were found that have FSH-stimulating activity (*2*) and have been shown to be the dimers of the inhibin β ($\beta_A\beta_A$ or $\beta_A\beta_B$) subunits (*3*). It has been suggested that these molecules be called activin A and activin AB to signify that they have biological effects opposite to inhibin (*3, 8*). It is not known what cell type, or types, secrete activin, but it has generally been assumed that activin and inhibin are secreted by the same cell type in the ovary (granulosa) and in the testis (Sertoli) (*2*). The control of processing that might lead to the production of one or the other hormone expressed in the same cell is not understood.

A clonal cell line derived from mouse testicular Leydig (TM3) cells (*9*) was screened by RNA blot analysis for the presence of sequences hybridizing with human cDNA probes for the α, β_A, and β_B subunits of inhibin. The TM3 cell line contained mRNA that hybridized with both the β_A (Fig. 1A) and, in a lesser amount, the β_B (*10*) probes. These two mRNAs were the same size as those from porcine ovary. No hybridization could be detected with the cDNA for the α subunit of inhibin under any conditions tried (Fig. 1B). These results suggested that TM3 cells express only the β mRNAs and may therefore be a source of activin. To determine whether these cells were capable of secreting a protein with the properties of activin, we tested conditioned medium from the TM3 cell line in a dispersed pituitary cell bioassay system (*8*). This medium elicited an increase in FSH, but not LH, release from the pituitary cell cultures compared to the cell-free control.

To confirm these results and rule out the possibility that the established cell line is expressing a gene not normally expressed in vivo, we prepared interstitial cell cultures from immature rat and pig testis that were enriched for Leydig cells. Sertoli cell–enriched cultures were prepared and assayed

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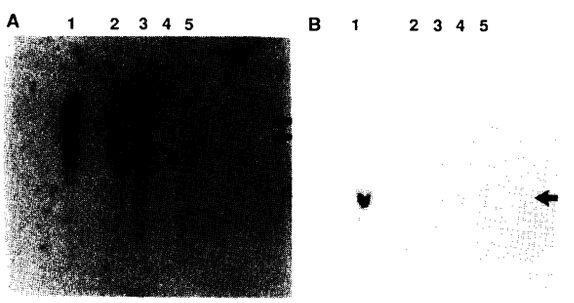

Fig. 1. RNA blot analysis of mRNA isolated from porcine follicles (lane 1), the TM3 Leydig cell line (lanes 2 and 3), and the TM4 cell line (a non-Leydig mouse testis line) (*9*) (lanes 4 and 5). Ten micrograms of total (lanes 1, 2, and 4) or polyadenylated (lanes 3 and 5) mRNA was loaded on each lane. The blots were hybridized with full-length cDNA probes for (**A**) the human inhibin β_A subunit or (**B**) the α subunit. The cDNA probes were prepared and hybridization was carried out as previously described (*5*). The TM4 did not show hybridization to either cDNA. Arrows indicate the position of porcine β_A mRNA [7.2 kb (top) and 4.5 kb (bottom)] in (A) and porcine α mRNA (1.3 kb) in (B).