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Transforming Growth Factor-Beta Protocols

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In Vitro Assays for Measuring TGF-β Growth Stimulation and Inhibition

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1. Introduction

Transforming growth factors (TGFs) were initially isolated from the conditioned medium of transformed cell lines through their ability to stimulate anchorage-dependent cells to form colonies in soft agar ***(1,2)***. The ability to proliferate in an anchorage-independent manner is still one of the best in vitro correlates with tumorigenicity. Subsequent studies demonstrated that the growth-promoting activity in the conditioned medium consisted of two unique peptides, TGF-α and TGF-β ***(3–5)***. Depending on the indicator cell line used, soft-agar colony growth could occur when TGF-α and TGF-β (i.e., NRK cells) or TGF-β alone (i.e., AKR-2B cells) were added to the serum-containing medium ***(6,7)***. This review will focus on TGF-β and cellular systems capable of responding in vitro to its growth modulatory activity independent of additional factors.

Transforming growth factor-β is a 25-kDa homodimeric protein representative of a family of molecules capable of regulating cell growth and differentiation ***(8–10)***. Three mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β 3, have been isolated ***(11)***. Although these molecules have similar and overlapping activity in the majority of in vitro assays, their role(s) in vivo appears to be quite distinct ***(12)***. This distinction becomes readily apparent when the phenotypes of TGF-β knockout mice are compared. For instance, whereas TGF-β1 null animals develop a multifocal inflammatory response and wasting following weaning, the lack of TGF-β2 or TGF-β3 results in a variety of developmental defects ***(13,14)***.

The cellular response to TGF-β is quite distinct, whereas mesenchymal cells are (in general) growth stimulated (both in vitro and in vivo), the majority of

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other cell types (i.e., epithelial, hematopoietic) are growth inhibited. It is unknown how a single growth factor, binding to the same set of receptors, can generate such divergent phenotypes as growth in soft agar, apoptosis, and/or growth arrest. Although studies on the growth-promoting activity of TGF-β have not recently generated as much interest as the growth-inhibitory response, a large body of literature exists documenting the importance of TGF-β in wound healing and various fibroproliferative disorders ***(15–18)***.

Although the approaches discussed in this chapter can be directly employed on any anchorage-dependent culture, they have primarily been utilized with mesenchymal cell cultures. Specifically, we will discuss methods for the following:

1. Thymidine incorporation
2. Autoradiography
3. Soft-agar colony formation
4. Morphological transformation

Although each of these assays can be readily modified to a variety of cell systems, this chapter will focus on two specific model systems: the AKR-2B cell line as a representative mesenchymal culture growth stimulated by TGF-β ***(19,20)***, and the Mv1Lu (CCL64) epithelial cell line, for which TGF-β acts as a late G1 phase growth inhibitor ***(21)***.

2. Materials

2.1. Cell Culture

1. Dulbecco's modified eagle medium (DMEM) (Life Technologies Inc., Gaithersburg, MD).
2. McCoy's 5A Medium (Life Technologies Inc.).
3. MCDB 402 (JRH Bioscience, Lenexa, KS).
4. Fetal bovine serum (Summit, Ft. Collins, CO).
5. Sea plaque agarose (FMC Bioproducts, Rockland, ME).
6. Transforming growth factor-beta (TGF-β): This can be obtained from a number of commercial sources. We have found all to be equally active.

2.2. DNA Synthesis

1. ^{3}H-Thymidine (64 Ci/mmol) (ICN, Costa Mesa, CA).
2. Methanol.
3. Emulsion (Kodak NTB2, Eastman Kodak, Rochester, NY).
4. Developer (D19) (Eastman Kodak, Rochester, NY).
5. Fixer: 75 g Na thiosulfate, 31.3 g K metabisulfite, water to 250 mL.
6. Hematoxylin or Giemsa (Fisher Scientific, Pittsburgh, PA).
7. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
8. Trichloroacetic acid (TCA): 10% (w/v) in water.

2.3. Soft-Agar Colony Growth

1. 1.6% sea plaque agarose is made in distilled water and autoclaved for 30 min. The liquefied agarose is then aliquoted (approx 60 mL) into sterile 125-mL glass bottles and stored at room temperature.
2. 2X serum-free DMEM is made according to the manufacturers suggestions using half the normal amount of water. The medium is sterilized through a 0.2-μm filter and stored at 4°C.
3. 35-mm sterile tissue culture dishes (warmed to 37°C).
4. Fetal bovine serum.

3. Methods

3.1. Thymidine Incorporation

This assay is based on the ability of TGF-β to modulate the incorporation of ^{3}H-thymidine in cultured cells. In general, the monolayer growth of most cell types is inhibited when TGF-β is simultaneously added to the serum-containing medium. Although conditions have been defined whereby TGF-β can stimulate mesenchymal cell growth, the response is usually weaker than that commonly observed with other mitogens ***(19)***.

A significant variable for all monolayer assays is the initial cellular seeding density. We find it is most convenient to report this based on the apparent usable growth area in the tissue culture dish/flask. The growth area reported by Cristofalo and Charpentier for various common tissue culture flasks and dishes is listed below ***(22)***:

T150 flask	150 cm^2
T75 flask	75 cm^2
T25 flask	25 cm^2
100-mm dish	64 cm^2
60-mm dish	22 cm^2
35-mm dish	9.6 cm^2
24-well dish	2.0 cm^2
96-well dish	0.32 cm^2 (approximately)

3.1.1. Epithelial Cells

3.1.1.1. Cycling Cultures

1. Mv1Lu cells (Mink Lung Epithelial Cells; CCL64) are plated at (1–2) × 10^4 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS). We routinely use 24-well dishes in a total volume of 1.0 mL.
2. Following 20–24 h at 37°C in a 5% CO_2 incubator, a 100X stock (10 μL) of TGF-β is directly added to duplicate wells for an additional 20–24 h. For most uses, the final TGF-β concentration ranges from 1.0 to 10.0 ng/mL (40–400 p*M*).
3. Add 10 μL of 100 μCi/mL ^{3}H-thymidine (64 Ci/mmol) for each 1.0 mL of medium and incubate at 37°C for 1–2 h. Final ^{3}H-thymidine concentration of 1.0 μCi/mL.

4. Remove (discard) labeled medium by aspiration and fix with 1.0 mL of 10% TCA per well for 10 min at room temperature.
5. Remove TCA (aspirate or dump out) and repeat TCA fixation (2X) described in **step 4**.
6. Aspirate TCA to dryness and solubilize in 300 μL (per 24 well) of 0.2 *N* NaOH containing 40 μg/mL sheared salmon sperm DNA.
7. Place on platform rocker and rock for 10–20 min at room temperature.
8. Take a 100-μL aliquot from each well of the 24-well plate, place in scintillation vial, and 5.0 mL scintillation fluid. A separate pipet tip should be used for each well (including duplicates).
9. Mix samples and count for 5 min. Shorter (i.e., 1 min) times can be used, however, if your counts are low, significant error can occur due to photoactivation.

3.1.1.2. Arrested/Restimulated Cultures

1. Mv1Lu cells are plated at 2×10^4 cells/cm^2 in DMEM supplemented with 10% FBS. We routinely use 24-well dishes in a total volume of 1.0 mL (4×10^4 cells/well).
2. Following 3 d growth, the medium is removed and the cultures rinsed 2X with 1.0 mL sterile PBS.
3. The second PBS rinse is removed and replaced with 1.0 mL DMEM containing 0.1% FBS for an additional 24 h incubation at 37°C.
4. Duplicate wells are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C to determine the basal (quiescent) incorporation (*see* **Subheading 3.3.** for stopping the incorporation). For the remaining wells, the medium is removed and the cultures are restimulated at 37°C with fresh DMEM supplemented with 10% FBS, 10 ng/mL epidermal growth factor (EGF), ± [TGF-β]. If you wish to determine a particular cell cycle "window" where TGF-β acts ***(21,23,24)***, 10 μL of a 100X TGF-β stock can be directly added to the FBS/EGF-containing medium at the appropriate times.
5. Following 20–24 h stimulation, the cultures are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C. To determine the minimum G1 transit time and/or rate of entry into S phase, cultures can be pulsed for 1–2 h at any time during the 24 h stimulation and the reaction stopped with ascorbic acid as described in **Subheading 3.3.**
6. Cultures are TCA fixed and processed as described in **Subheading 3.1.1.1, steps 4–9**.

3.1.2. Mesenchymal Cells

3.1.2.1. Cycling Cultures

1. We routinely use AKR-2B cells as a mesenchymal model. Similar studies can be performed on Balb/c-3T3, 10T1/2, NIH, and so forth murine fibroblasts with minimal changes (determined empirically).
2. All steps are performed as described in **Subheading 3.1.1.1.** with the exception that 5%-FBS supplemented McCoy's 5A medium is used. Although DMEM can be used, we have found that McCoy's 5A (Life Technologies) medium supports the continued passage of AKR-2B cells better.

3.1.2.2. Arrested/Restimulated Cultures

1. AKR-2B cells are plated at 2×10^4 cells/cm^2 in McCoy's 5A medium (Life Technologies) supplemented with 5% FBS. We routinely use 24-well dishes in a total volume of 1.0 mL (4×10^4 cells/well).
2. Following 2 d growth, the medium is removed and the cultures rinsed 2X with 1.0 mL sterile PBS.
3. The second PBS rinse is removed and replaced with 1.0 mL serum-free MCDB 402 for an additional 48 h incubation at 37°C. MCDB 402 is an outstanding medium for serum-free culture ***(25)***. Many cells show essentially no change in viability following 1–2 wk incubation in the absence of serum.
4. Duplicate wells are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C to determine the basal (quiescent) incorporation (*see* **Subheading 3.3.** for stopping the incorporation). For the remaining wells, the medium is removed and cultures restimulated at 37°C with fresh McCoy's 5A medium (or DMEM) supplemented with the appropriate serum/growth factor "cocktail" ± [TGF-β].
5. Following 20–24 or 40–48 h stimulation, the cultures are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C. The response of mesenchymal cells in monolayer to TGF-β has been controversial. There have been reports of normal growth stimulation, delayed stimulation presumably due to autocrine activity of an induced mitogen, as well as growth inhibition. To determine the minimum G1 transit time and/or rate of entry into S phase, cultures can be pulsed for 1–2 h at any time during the 24-to 48-h stimulation and the reaction stopped with ascorbic acid (*see* **Subheading 3.3.**).
6. Cultures are TCA fixed and processed as described in **Subheading 3.1.1.1., steps 4–9**.

3.2. Autoradiography

1. Cells are plated and/or arrested as described in **Subheading 3.1.1.1., 3.1.1.2., 3.1.2.1., or 3.1.2.2.**

2a. For cycling cultures, 5.0 μCi/mL ^{3}H-thymidine is added for 2–4 h at 37°C during the final 2–4 h prior to fixation. Remember to pulse cultures for a similar time prior to addition of TGF-β to obtain the 0-h control.

2b. Quiescent restimulated cultures can be similarly pulsed as described in **step 2a** at the end of the experiment or the label can be present continuously for the course of the study.

3. The medium is aspirated, the cells are washed one to two times with PBS, and the cultures fixed with two 20-min applications of 100% methanol (10% TCA can be used, but we find that methanol preserves the cellular structure slightly better). The PBS and methanol applications can be done by simply dumping the medium out and gently pouring.
4. Following the final methanol fixation, the plates are gently washed in water three to five times. A hand-held eye wash works well, or simply dunk the plates in a beaker of water. Again, the water is removed by pouring/shaking into the sink.
5. The excess water is removed and the plates are air-dried.

6a. Go to the dark room and add a *thin* film of emulsion to the entire well. We use Kodak NTB2 diluted equally (w/v) with water (*see* **Subheading 3.3.**).
6b. Adding emulsion is tricky. For microtiter and 24-well plates, a little (i.e., 50–500 μL) is added to each well to ensure complete coating and the excess removed by a hard shake. For larger plates, a few milliliters (i.e., 2–5 mL) are added, the plate is swirled to cover, and the excess is directly added to the next plate, where the process is repeated (a Pasteur pipet may be needed to obtain proper coating).
7. The cultures are placed in a light-tight container (a cookie tin or Tupperware container wrapped in foil works well) over a layer of Drierite (Fisher Scientific) for 2–4 d at room temperature (or 4°C).
8. Develop autoradiography in the darkroom. Many chemicals will work, but be careful if you buy a fixer that it is not too harsh. This will work:
   a. D19 Developer - 4 min; remove.
   b. Water wash (gently).
   c. Fixer - 2 min; remove.
      75 g Na thiosulfate, 31.3 g K metabisulfite, bring to 250 mL with water.
   d. Water wash (gently).
9. Counterstain with Giemsa or hematoxylin (Fisher Scientific) for approximately 15 min (determine empirically). Pour stain off, wash excess with water, and air-dry.
10. Count (or better yet, get someone else to count them for you) labeled/total nuclei in representative field(s) using a 10× to 20× objective.

3.3. Additional Comments

1. A common technical problem is how to utilize a single plate while stopping wells at distinct times (i.e., when determining the kinetics of G1 traverse and entry into S phase). Fixatives such as TCA are problematic because of the potential for fume carryover to adjacent wells. One easy method to overcome this is to use an organic acid such as ascorbic acid for fixation ***(26)***. A 1.0 *M* stock (in water) of the free acid (not the salt) is prepared and 300 μL is added for each 1.0 mL of culture medium. This will stop any incorporation and the plate can now be placed back into the incubator. At the end of the experiment, the entire plate can now be TCA fixed and processed appropriately.
2. Aliquots (10 mL) of the 1.0 *M* ascorbic acid are stored at –20°C. Once thawed, a sample can be maintained at room temperature for approx 1 wk (it will start to turn brownish).
3. The emulsion for autoradiography needs to be dissolved in a 50–55°C water bath. Once you get a stock diluted (i.e., 100 mL), it is convenient to aliquot the emulsion (i.e., 5–10 mL), wrap the tubes in foil, and store at 4°C. A tube(s) can then be used and any remaining discarded. Although the excess can be reused, this sometimes results in high-background problems.
4. Autoradiography with microtiter plates is difficult. An additional way to process those wells is (following fixation) to score the back of the well, use an appropriate size punch and hammer to knock the well out, and glue (use clear glue) the well-scored side down on to a microscope slide. Two rows of six wells can be

placed on a slide. The slides can then be dipped in emulsion, exposed, and developed as discussed in **Subheading 3.2.** Although initially more difficult, this method is preferred.

3.4. Colony Formation in Soft Agar

Transforming growth factor-β was initially identified by its ability to stimulate anchorage-dependent mesenchymal cells to grow in an anchorage-independent manner. The ability of anchorage-dependent cells to form colonies in soft agar is one of the best in vitro correlates with tumorigenicity. Although some cell lines (i.e., AKR-2B) only require the addition of TGF-β to the serum-supplemented medium ***(6)***, other lines (i.e., NRK) also need exogenous EGF (or TGF-α) plus TGF-β for optimal growth in soft agar ***(7)***. Finally, whereas the majority of studies presently focus on TGF-β's growth inhibitory actions, the in vivo growth-promoting role that TGF-β contributes during wound healing or in the pathogenesis of fibrotic disease(s) should not be underestimated ***(15–18)***.

3.4.1. Bottom Plugs

1. Bottom plugs consist of 1X DMEM supplemented with 10% FBS and 0.8% agarose. You need 1.0 mL for each 35-mm plate. Example: If 20 plates are required, combine 10 mL 1.6% agarose, 2.0 mL FBS, and 8.0 mL 2X DMEM. First, combine the serum and DMEM and set in a 37°C water bath to warm; second, microwave the agarose to liquefy; third, when the glass bottle is cool to your skin, mix with the media and serum and pipet 1.0 mL into the required number of 35-mm plates.
2. One milliliter does not flow easily over the plate bottom, you must tilt the plate while pipetting to ensure complete covering. These plates may be prepared 1 d in advance. After solidifying at room temperature, store at 37°C in a 5% CO_2 incubator.

3.4.2. Top Plugs

1. Top plugs consist of 1X DMEM supplemented with 10% FBS, 0.4% agarose, cells, ± TGF-β or other test reagents. The cell concentration can range from 5.0×10^3 to 2.0×10^4 cells/mL. If the cell concentration is too high ($>2.0 \times 10^4$ cells/mL), false positives can be obtained as a result of cell aggregation. We routinely use AKR-2B cells at 1.0×10^4 cells/mL (addition of cells discussed in **steps 3** and **4**).
2. For 35-mm plates, you need 1.0 mL/plate. Each sample is done in triplicate (total volume 4.0 mL) using a 17 × 100-mm or 15-mL conical tube.
3. Each tube will now receive 0.4 mL FBS and 2.0 mL 2X DMEM. Add 4.0×10^4 cells ± TGF-β (final concentration of 3–10 ng/mL) or any other test reagent(s) in a final volume of 0.6 mL 1X DMEM. Mix and place in a 37°C water bath. Be sure to have plates that do not receive TGF-β to determine spontaneous colony formation.
4. Microwave the 1.6% agarose to liquefy and cool until the bottle is not uncomfortable to check. This is the most critical part of the assay; you need to have agarose

warm enough so the top plugs do not solidify too soon, yet cool enough so you do not fry your cells.
5. Using a 5-mL pipet, pipet 1.0 mL of agarose into one tube and mix by pipetting up and down. Quickly pipet 3.0 mL, dispense 1.0 mL/plate, and tilt the plate to ensure complete covering. Do not add the agarose to a number of tubes prior to plating. This will likely result in the mixture prematurely solidifying (this can be avoided by placing the bottom plugs in a 37°C for 15–30 min prior to addition).
6. Let plates solidify at room temperature and then place at 37°C in a 5% CO_2 incubator for 1–2 wk.

3.4.3. Analysis

1. Quantitation is most easily performed using a computerized image analysis system where a defined size can be determined to represent significant colony growth. We have previously used an Omnicon Image Analyzer (BioLogics) with a threshold of 50 μm for AKR-2B cells. Other investigators ***(27)*** have utilized EagleSight analysis software (Stratagene, La Jolla, CA) following staining for 20 h at 37°C in a 1.0 μg/mL solution (in water) of iodonitrotetrazolium violet.
2. Because the above systems are quite expensive, an alternative method is to use a microscope with an eyepiece grid. The entire plate is analyzed and cell clusters of greater than 10 cells are counted as positive.
3. It is also possible to employ a qualitative analysis of the data by simply photographing representative fields on a 10× bright field.

3.5. Morphological Transformation

Cytoskeletal alterations were one of the earliest cellular findings associated with viral transformation ***(28,29)***. It was subsequently found that TGF-β modulated the expression of various cytoskeletal and extracellular matrix genes ***(30–32)***. Coincident with these effects on gene expression, TGF-β induces a morphologic change in mesenchymal cultures similar to that observed during the growth of transformed cell lines ***(33,34)***. The following assay was designed to optimize that phenotype in AKR-2B cells as a model of cytoskeletal rearrangement.

1. AKR-2B cells are plated in 60-mm culture dishes at a density of 1.36×10^4 cells/cm^2 in 4.0 mL (7.5×10^4 cells/mL) of McCoy's 5A medium supplemented with 5% FBS.
2. Incubate at 37°C for 2–4 d until confluence.
3. Wash 2X with 4.0 mL sterile PBS.
4. Remove the PBS and add 4.0 mL serum-free MCDB 402.
5. Incubate at 37°C for 2 d.
6. Remove the medium and replace with 2.0 mL serum-free MCDB 402 ± any test reagent (i.e., TGF-β at 10 ng/mL). Place back at 37°C.
7. Twenty-four hours later, directly add fresh TGF-β (10–100 μL) to a final concentration of 10 ng/mL.

8. Continue incubation at 37°C for an additional 24 h.
9. Remove the medium, wash 1X with PBS, add 2.0 mL PBS and photograph at 20X phase contrast.

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2

Measurement of Active TGF-β Generated by Cultured Cells

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1. Introduction

1.1. TGF-β Latency and Activation

The transforming growth factors-β (TGF-βs) constitute a family of potent regulators of cellular differentiation, proliferation, migration, and protein expression ***(1,2)***. Three isoforms of TGF-β have been described in mammals: TGF-β1, 2, and 3 ***(3–5)***. Most cell lines and tissues secrete TGF-β as a large latent complex formed by three components: TGF-β, LAP (latency-associated protein), and LTBP (latent TGF-β binding proteins). TGF-β is noncovalently associated to its prodomain LAP ***(6–8)***, and LAP is disulfide-bonded to LTBP ***(9)***. Four LTBPs (LTBP-1, 2, 3, and 4) have been described ***(10–14)***. Mature TGF-β must be released from the complex to bind to its high-affinity receptor and elicit its biological functions ***(15)***. This process, called TGF-β activation, appears to be a critical step in the control of TGF-β activity ***(16)***. An additional regulatory step involved in the activation process is the LTBP-mediated incorporation of latent TGF-β into the extracellular matrix ***(2)***. Activation of latent TGF-β has been described in various cell systems ***(17–19)***. However, the molecular mechanisms involved in extracellular TGF-β activation are not fully understood. It also remains to be elucidated whether latent TGF-β incorporation into the extracellular matrix regulates TGF-β activation in a positive or negative manner ***(2)***.

1.2. Detection of Active TGF-β

The availability of sensitive, specific, and quantitative assays for the detection of mature TGF-β is of fundamental importance in studying TGF-β

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activation. The purpose of this chapter is to describe some of the assays used in our laboratory to measure active TGF-β in cell systems. These assays are as follows:

1. Wound assay for bovine aortic endothelial cell migration.
2. Cellular plasminogen activator assay for TGF-β.
3. Mink lung-cell growth-inhibition assay.
4. Mink lung epithelial cells luciferase assay.

In all these assays, the active TGF-β generated by the test cells induces a known and measurable biological response in the reporter cells such as inhibition of endothelial cell migration ***(20,21)***, inhibition of epithelial cell proliferation ***(22)***, decreased plasminogen activator activity ***(23)***, and increased production of plasminogen activator inhibitor-1 ***(24)***. All of these assays can be used to measure active TGF-β released by the test cells into their medium.

Only a few primary cells and established cell lines secrete significant amounts of active TGF-β into their culture medium when properly treated. Some examples are treatment of keratinocytes with retinoids ***(25)*** or vitamin D analogs ***(26)***, treatment of cancer cells or normal fibroblasts with antiestrogens ***(27,28)***, and treatment of MG-63 osteosarcoma cells with corticosteroids ***(29,30)***. Otherwise, little, if any, soluble active TGF-β is generated by most cultured cells. The absence of detectable levels of active TGF-β in the medium of TGF-β-producing cells is a common situation. However, the lack of active TGF-β in a cell culture supernatant does not necessarily mean lack of TGF-β activation. This may be because of two reasons. First, in some cases, TGF-β activation occurs at the cell surface ***(17,31,32)***, generating a high local concentration of active TGF-β. Second, active TGF-β is cleared from solution by binding to cell-surface receptor and/or to the extracellular matrix. As a result, only a small fraction may be released into the medium and therefore diluted to undetectable levels. High local concentration of active TGF-β can be detected by reporter cells cocultured with the activating cells ***(17,33)***. A useful TGF-β assay must be both sensitive and specific. Neutralizing antibodies to TGF-β should be included to verify that there are no other factors present that may affect the assay. Addition of isoform-specific neutralizing antibodies and use of the appropriate standard curves will allow quantification of specific TGF-β isoforms. When analyzing the effect of a treatment on TGF-β activation, one must determine if increased active TGF-β is the result of increased activation of latent TGF-β or increased production of total (active plus latent) TGF-β without any change in the latent versus active TGF-β ratio. In most TGF-β assays, the amount of total TGF-β released into the culture medium can be measured upon activation of the latent fraction by either acidification ***(31)*** or heat treatment ***(34)***.

2. Materials

2.1. General

1. Minimum essential medium (αMEM), store at 4°C.
2. Dulbecco's modified Eagle medium (DMEM), store at 4°C.
3. Fetal calf serum (FCS), store at –20°C, keep at 4°C after thawing.
4. Bovine serum albumin (BSA), store at 4°C.
5. Penicillin–streptomycin–L-glutamine (PSG) stock (100×): 20 g/L strepto mycin, 50×10^6 U/L penicillin G, 29.2 g/L L-glutamine. Filter sterilize and store aliquots at –20°C. Keep at 4°C after thawing.
6. Phosphate-buffered saline (PBS) pH~7.3: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4. Filter sterilize and store at 4°C.
7. Trypsin solution pH 7.2: 0.25% trypsin, 1 m*M* EDTA. Filter-sterilize and store aliquots at –20°C. Keep at 4°C after thawing.
8. Recombinamt TGF-β (rTGF-β) stock solution: 5 m*M* HCl, 0.1% BSA, 2 μg/mL TGF-β. Store at 4°C.
9. Neutralizing anti-TGF-β antibodies and nonimmune IgG. Store aliquots at –30°C. Keep at 4°C after thawing.
10. Control medium (serum-free medium): To avoid effects of serum factors, most experiments are conducted in the absence of serum. Serum-free medium contains αMEM or DMEM, depending on the cell type used in each assay, 0.1% BSA and 1× PSG. Filter sterilize and store at 4°C.
11. Test cells conditioned medium (*see* **Notes 1–4**): (a) Plate the cells at subconfluence in regular growth medium and let them attach at 37°C for 2–4 h; (b) wash twice with PBS, (c) add serum-free medium and incubate at 37°C for 24 h; (d) collect the medium and centrifuge to remove cell debris. The conditioned medium is ready to be tested for the presence and levels of total and active TGF-β.
12. Acid-or heat-activated conditioned medium (*see* **Notes 5** and **6**). Acidification: (a) Acidify the conditioned medium to pH 2 with 1 *M* HCl; (b) incubate 1 h at room temperature; (c) neutralize with 1*N* NaOH. Use immediately. Heat treatment: (a) incubate the conditioned medium for 10' at 80°C; (b) let the medium cool down to 37°C. Use immediately.

2.2. Wound Assay for BAE Cell Migration

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH_2O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Rigid razor blade (*see* **Note 7**).
4. Absolute methanol.
5. 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetyl LDL (DiI-acetyl-LDL).

6. 3% Formaldehyde in PBS: Formaldehyde is usually obtained as a 37% solution in H_2O. Dilute the stock solution 1:12.3 in PBS. Formaldehyde vapors are toxic; prepare the solution in a chemical hood.
7. Light microscope with ocular grid.

2.3. Mink Lung-Cell Growth-Inhibition Assay

1. CCL-64 cells (American Type Culture Collection, Rockville, MD).
2. ^{3}H-Thymidine (^{3}H-TdR), 40-60 Ci/mmol.
3. ^{125}I-Deoxyuridine (^{125}I-UdR), 5 Ci/mg.
4. 3:1 (v/v) Methanol–acetic acid.
5. 80% methanol.
6. 0.5% trypsin.
7. 1% sodium dodecyl sulfate (SDS).
8. Liquid scintillation counter.
9. 1*N* NaOH.
10. Gamma counter.

2.4. Cellular Plasminogin Activator Assay

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH_2O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Lysis buffer: 0.1 *M* Tris–HCl, pH 8.1, 0.5% Triton X-100.
4. Bovine fibrinogen.
5. 0.1X PBS: 13,7 m*M* NaCl, 0.27 m*M* KCl, 0.43 m*M* Na_2HPO_4, 0.14 m*M* KH_2PO_4.
6. ^{125}I-Fibrinogen, prepared by the iodine chloride method ***(35)***.
7. 2.5% FCS in αMEM, prepare freshly.
8. Assay buffer: 0.1 *M* Tris–HCl, pH 8.1, 250 μg/mL BSA, 8 μg/mL plasminogen. Prepare freshly.
9. Urokinase stock: 0.1 *M* Tris–HCl, pH 8.1, 0.1% BSA, 1000 U/mL urokinase. Store 10-μL aliquots at –20°C. Just before use, diluted with 5 mL of Tris–HCl, 0.1 *M*, pH 8.1, 0.1% BSA. Keep the dilutions on ice. Urokinase activity is not stable to repeated freezing and thawing.
10. Plasminogen. Purification of plasminogen is carried out at 4°C using a 100-mL lysine–Sepharose column per 500 mL of serum:
    a. Equilibrate the column with PBS.
    b. Load the serum.
    c. Wash with at least three column volumes of 0.3 *M* potassium phosphate, pH 7.4, 2 m*M* EDTA; wash the column until the optical density $(OD)\lambda_{280}$ returns to the basal value.
    d. Elute the plasminogen with 0.2 *M* ε-aminocaproic acid in 0.1 *M* potassium phosphate, pH 7.4; collect 5-mL fraction and read the $OD\lambda_{280}$ to follow the elution profile (*see* **Note 8**).

e. Pool the eluted proteins and dialyze against PBS to remove the ε-amino-caproic acid.
f. Measure the ODλ_{280} (OD of 1.7 units = 1.0 mg/mL of plasminogen), aliquot, and store at –20°C.

11. Gamma counter.

2.5. MLEC Luciferase Assay

1. Mink lung epithelial cells (MLEC) permanently transfected with the expression construct p800neoLUC ***(36)***.
2. Geneticin stock solution (Invitrogen, Carlsbad, CA): 80 mg/mL in PBS. Fillter-sterilize and store at –20°C.
3. Lysis buffer (Analytical Luminescence, San Diego, CA). Dilute 1:3 with dH_2O the 3X stock solution. Prepare freshly.
4. Assay buffer. Prepare freshly from the following stock solutions: 5X luciferin buffer [1 *M* tricine, 5.35 m*M* $(MgCO_3)_4Mg(OH)_2$, 13.35 m*M* $MgSO_4$, 0.5 m*M* EDTA, 166.5 m*M* DTT]; 50X ATP (37.5 m*M*); 20X luciferin (16 m*M*). Keep luciferin in the dark. (Lucifrerin is rapidly oxidized by exposure to light.) Store stock aliquots at –30°C.
5. Luminometer.

3. Methods

3.1. Wound Assay for BAE Cell Migration

This assay is based on the ability of TGF-β to inhibit cell migration in "wounded" monolayer cultures of BAE cells ***(20,21)***. The number of cells that migrate across the original edge of the wound is inversely proportional to the concentration of TGF-β present in the conditioned medium.

3.1.1. Cell Culture

1. Grow BAE cells on gelatin-coated dishes in αMEM containing 10% FCS and 1X PSG.
2. Use cells at early passages (not after passages 15–20).

3.1.2. Wound Assay

Portion of a confluent culture of BAE cells is removed by mechanical abrasion using a rigid razor blade ***(37)***.

1. Sterilize the razor blade in the pilot light of a Bunsen burner and let it cool down.
2. Use a surgical hemostat to manipulate the razor blade. Press the razor blade down onto the plate to cut the cell monolayer and to lightly mark the original edge of the wound by scoring the plastic surface (*see* **Notes 7** and **9–11**).
3. Gently move the blade to one side to remove part of the cell monolayer.
4. Wash twice with PBS to remove loose cells.
5. According to the experimental design, add the following:
   a. Control medium to determine the basal level of cell migration.

b. Control medium containing increasing amounts of rTGF-β to generate a standard curve; this assay can be used to detect concentrations of TGF-β as low as 0.4 p*M* ***(32)***.
c. Conditioned medium from the experimental cultures to measure active TGF-β.
d. Acid-or heat-activated conditioned medium to measure total (active plus latent) TGF-β.
e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the migration inhibition.

6. Incubate at 37°C for 16–20 h.
7. Remove the medium and wash once with PBS.
8. Fix the cells with absolute methanol for 10–15' at room temperature.
9. Count the number of cells that have migrated more than 125 μm from the wound edge in seven successive 125-μm increments. The cells present in the first 125 μm segment are not included in the calculation in order to exclude those cells which moved across the origin before TGF-β had an effect ***(38,39)***. Cells are counted at 40X magnification using a light microscope with an ocular grid.
10. Data are presented as percent of migration observed in the control wound. For each experimental condition, the number of migrating cells is counted in four to six random fields from each of two replicate dishes and the mean value is used to calculate the percent of migration inhibition.

3.1.3. Coculture Assay

1. Immediately after wounding, the second cell type is suspended in serum-free medium and inoculated into the culture dish (*see* **Note 12**).
2. Incubate at 37°C for 16–20 h.
3. Count the migrating BAE cells as in the standard wound assay (*see* **Note 13**).

3.2. Mink Lung-Cell Growth-Inhibition Assay

CCL-64 mink lung epithelial cells have been shown to be extremely sensitive to growth inhibition by TGF-β ***(40)***. A very sensitive and specific assay for TGF-β has been described by Danielpour and colleagues ***(22)***. They have shown that CCL-64 cells plated in DMEM containing 0.2% FCS are half-maximally growth inhibited by about 0.5 p*M* of TGF-β after 22 h of treatment. Because of this sensitivity, conditioned media can be assayed without concentration. Because other growth factors such as insulin, EGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been shown not to stimulate or inhibit CCL-64 cell proliferation, this assay is relatively specific for TGF-β ***(22)***.

3.2.1. Cell Culture

1. Grow CCL-64 cells in the high-glucose formulation of DMEM supplemented with 10% FCS and 1X PSG.
2. Pass the cells at a seed density of 5×10^5 cells/75 cm^2 T-flask at 3 d intervals.

3.2.2. Growth-Inhibition Assay

CCL-64 cells in logarithmic growth phase are used to initiate the growth inhibition assay.

1. Trypsinize and suspend the cells in 10 mL of DMEM 10% FCS
2. Centrifuge the cells at 500g for 5'.
3. Wash the pellet once with 10 mL of DMEM containing: 0.2% FCS and 1X PSG.
4. Resuspend the cells in the same medium.
5. Count and dilute the cells to a final concentration of 10^6 cells/mL.
6. Seed 0.5 mL/well of cell suspension in 24-well plates.
7. Let the cells attach at 37°C for 2 h.
8. Remove the medium and add the following according to the experimental design:
   a. Control medium to determine the basal level of proliferation.
   b. Control medium containing various concentrations of rTGF-β to generate a standard curve; this assay can be used to measure TGF-β in the range 0.08–2.4 p*M* ***(41)***.
   c. Conditioned medium from the experimental culture to measure active TGF-β.
   d. Acid- or heat-activated conditioned medium to measure total (latent plus active) TGF-β.
   e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the growth inhibitory response.
9. Incubate at 37°C for 22 h.
10. Remove the medium and pulse the cells with 0.25 mCi (40–60 Ci/mmol) of ^{3}H-TdR or 0.25 mCi (5 Ci/mg) of ^{125}I-UdR diluted in DMEM, 0.2% FCS, 1X PSG for 2 h at 37°C.
11. Remove the radioactive medium.
12. Fix the cells with 1 mL of methanol–acetic acid (3:1 v/v) for at least 1 h at room temperature.
13. Wash the wells twice with 1 mL of 80% methanol.
14. If ^{3}H-TdR is used, incubate with 250 µL of 0.5% trypsin for 30' at 37°C; solubilize the radioactivity with 250 µL of 1% SDS; measure the radioactivity by liquid scintillation counting.
15. If ^{125}I-UdR is used: lyse the cells with 1 mL of 1*N* NaOH for 30' at room temperature; ^{125}I-UdR is counted in a γ-counter.

3.3. Cellular PA Assay for TGF-β

This assay is based on the observation that TGF-β suppresses plasminogen activator (PA) activity of endothelial cells ***(23)***. The inhibitory effect of TGF-β is predominantly the result of the increased synthesis of plasminogen activator inhibitor-1 (PAI-1) ***(42)***.

Plasminogen activator activity in cell extracts or conditioned media can be measured using the ^{125}I-fibrin assay ***(43)***. Samples are tested in the presence of a known amount of plasminogen in ^{125}I-fibrin-coated plates. The PA present in

the test samples converts plasminogen into plasmin, and plasmin degrades fibrin. The amount of ^{125}I-fibrin degradation products released into the supernatant correlates with the levels of PA activity present in the sample. PA activity can be quantitated using a standard curve generated with purified urokinase plasminogen activator (uPA).

3.3.1. Cell Culture

Bovine aortic endothelial cells are grown as described in **Subheading 3.1.1.**

3.3.2. PA Assay

1. Grow BAE cells to confluence in 96-well plates in complete growth medium.
2. Remove the medium and wash the cells with PBS.
3. Add the following in duplicate:
   a. Control medium to determine the basal level of PA activity
   b. Control medium containing increasing concentration of rTGF-β to generate a standard curve; this assay can be used to measure TGF-β in the 0.08–2.4-p*M* range ***(41)***.
   c. Conditioned medium from the experimental medium to measure active TGF-β.
   d. Acid-or heat-activated conditioned medium to measure total (latent plus active) TGF-β.
   e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the PA inhibitory response.
4. Incubate the cells at 37°C for 12 h.
5. Remove the medium and wash twice with ice-cold PBS.
6. Lyse with 50 µL/well of lysis buffer.
7. Determine protein concentration.
8. Measure PA levels in the cell extracts using the ^{125}I-fibrin plate assay (*see* **Note 14**).

3.3.3. ^{125}I-Fibrin Plate Assay

3.3.3.1. Preparation of ^{125}I-fibrin plates ***(43)***

1. Dilute bovine fibrinogen in warm (37°C) 0.1X PBS. Do not mix or vortex. Fibrinogen is diluted such that a volume can be spread over the bottom of the well to give a concentration of 10 µg/cm^2. If using 24-well plates, add 250 µL/well of 120 µg/mL fibrinogen solution.
2. Add ^{125}I-fibrinogen to bring the solution to approximately 160,000 counts per minute (cpm)/mL (40,000 cpm/well).
3. Aliquot 250 µL to each well, making sure that the entire bottom surface is covered.
4. Dry the open plates overnight under the hood.
5. Add 250 µL/well of medium containing 2.5% FCS. Fibrinogen is cleaved to fibrin by the action of thrombin present in serum.
6. Incubate at 37°C for 3 h.
7. Remove medium, wash twice with dH_2O, and store dry plates at 4°C.