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Regulation of smooth muscle α-actin expression and hypertrophy in cultured mesangial cells

LEAH A. STEPHENSON, LISA B. HANEY, ISA M. HUSSAINI, LARRY R. KARNS, and WILLIAM F. GLASS II

Department of Pathology, Eastern Virginia Medical School, Norfolk, and Department of Pathology, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA

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Background. Mesangial cells during embryonic development and glomerular disease express smooth muscle α-actin (α-SMA). We were therefore surprised when cultured mesangial cells deprived of serum markedly increased expression of α-SMA. Serum-deprived mesangial cells appeared larger than serum-fed mesangial cells. We hypothesized that α-SMA expression may be more reflective of mesangial cell hypertrophy than hyperplasia.

Methods. Human mesangial cells were cultured in medium alone or with fetal bovine serum, thrombin, platelet-derived growth factor-BB (PDGF-BB) and/or transforming growth factor-β_1 (TGF-β_1). α-SMA expression was examined by immunofluorescence, Western blot, and Northern blot analysis. Cell size was analyzed by forward light scatter flow cytometry.

Results. α-SMA mRNA was at least tenfold more abundant after three to five days in human mesangial cells plated without serum, but β-actin mRNA was unchanged. Serum-deprived cells contained 5.3-fold more α-SMA after three days and 56-fold more after five days by Western blot. Serum deprivation also increased α-SMA in rat and mouse mesangial cells. The effects of serum deprivation on α-SMA expression were reversible. Mesangial cell mitogens, thrombin or PDGF-BB, decreased α-SMA, but TGF-β_1 increased α-SMA expression and slowed mesangial cell proliferation in serum-plus medium. Flow cytometry showed that serum deprivation or TGF-β_1 treatment caused mesangial cell hypertrophy. PDGF-BB, thrombin, or thrombin receptor-activating peptide blocked hypertrophy in response to serum deprivation.

Conclusions. We conclude that increased α-SMA expression in mesangial cells reflects cellular hypertrophy rather than hyperplasia.

Smooth muscle α-actin (α-SMA) is expressed in mesangial cells in normal development [1–3] and in experimental [4–9] and human [10, 11] glomerular disease, but little or no α-SMA is expressed in normal adult mesangial cells *in vivo*. Consequently, expression of α-SMA is considered a marker of mesangial cell activation [7, 10–14]. Hyperplasia and hypertrophy are two specific mesangial cell responses that may result from mesangial cell activation [7]. Some studies have shown that α-SMA correlates with mesangial cell proliferation [4, 6, 9, 10], but other studies have found exceptions to this correlation [8, 11, 15, 16].

Serum contains extracellular matrix proteins [17–19], platelet factors [20–28], and other agents derived from activation of coagulation (such as, thrombin) that potentially regulate mesangial cell function in variety of ways. In fact, products of platelet activation during coagulation are responsible for the ability of serum to cause cell proliferation [21, 22]. Many of these same factors, either released by platelets or produced by glomerular cells, are thought to be important mediators of disease and responsible for mesangial cell activation.

Factors responsible for the expression of α-SMA in glomerular disease are poorly understood. α-SMA expression was associated with increased expression of platelet-derived growth factor (PDGF) and/or PDGF receptor in some investigations [6, 7, 29]. *In vitro* studies are also limited. In one study, growth arrest of rat mesangial cells for seven days in serum-free medium did not change the percentage of α-SMA positive cells [30]. In another study, serum stimulated transcription of a reporter gene fused to a sequence spanning −894 to +1 of the α-SMA promoter in transfected rat mesangial cells [12], but the effect of serum directly on α-SMA was not determined. In another study, the role of the serum response element in regulating α-SMA gene expression in rat mesangial cell hillocks was recently examined [31]. These investigators found that both the expression of smooth muscle actin and the activity of a β-galactosidase reporter were decreased in hillocks compared to surrounding cells. α-SMA mRNA and reporter mRNA were also decreased when cells were embedded in collagen gels. All of these studies suggested that serum should increase α-SMA in routinely cultured mesangial cells.

Recently, we showed that human mesangial cells survive

Key words: human development, cytoskeleton, cell size, microfilaments, flow cytometry.

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for several weeks in medium without serum or added growth factors [32]. We expected serum deprivation to cause a loss of α-SMA expression. In contrast to our expectations, serum deprivation increased α-SMA expression by an order of magnitude. The effects of PDGF-BB, thrombin and transforming growth factor β_1 (TGF-β_1) were also investigated. PDGF-BB and thrombin are mitogenic in human mesangial cells [33] and, like serum, inhibited α-SMA expression. TGF-β_1 is anti-proliferative in mesangial cells [34] and stimulated α-SMA expression. By flow cytometry, increased cell size accompanied increased α-SMA expression. These observations suggest that increased α-SMA expression in disease may be evidence of mesangial cell hypertrophy rather than hyperplasia.

METHODS

Materials

Antibody to α-SMA (clone 1A4) was from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant PDGF-BB, recombinant TGF-β_1 and monoclonal antibody to PDGF-BB were from R&D Systems (Minneapolis, MN, USA). Thrombin was a gift of Dr. J. Fenton (Wadsworth Center Labs, Albany, NY, USA). Complementary DNA probes used in these studies were α-SMA, graciously given by Dr. C. Chandra Kumar (Schering-Plough Research Institute, Kenilworth, NJ, USA) and β-actin [35] (ATCC #37997), glyceraldehyde phosphate-3-dehydrogenase (GAPD) [36] (ATCC#57091), and 18S ribosomal RNA [37] (ATCC# 77242) from The American Type Culture Collection (Rockville, MD, USA).

Cell culture

Human mesangial cells were isolated as described [32]. The cells were routinely grown in RPMI 1640 with fetal bovine serum (FBS 5:1) without antibiotics or other additives and were used during the first 10 passages. Serum-free cells were prepared by washing three times and resuspending in RPMI 1640 prior to plating. Rat mesangial cells were isolated by us as described [32] from the kidneys of Sprague-Dawley rats sacrificed for hepatectomy [38] in the laboratory of Dr. Steven L. Gonias (Department of Pathology, University of Virginia Health Sciences Center). Rat mesangial cells were positive for Th1.1 by both indirect immunofluorescence and Western blot. Mouse mesangial cells from non-diabetic NOD mice [39] were a gift of Dr. Oleh Pankewycz (Department of Medicine, University of Virginia Health Sciences Center).

Typical plating conditions included: (*1*) in RPMI 1640 with 16.7% (5:1 dilution) serum, (*2*) in RPMI 1640 without other additives, but onto serum-coated substrata, or (*3*) in RPMI 1640 alone onto non-coated substrata. Cells were plated on serum-coated (condition 2) and non-coated (condition 3) coverslips to compare the effects of different substrata on α-SMA expression. Under both conditions, cells become fully growth arrested after about one week [32]. On serum-coated substrata, adhesion at focal contacts is mediated via vitronectin receptors, but on non-coated substrata adhesion at focal contacts is mediated by fibronectin receptors [32].

RNA preparation and Northern blot

Total RNA was extracted using TRIzol reagent from Gibco BRL (Gaithersburg, MD, USA) and analyzed using ultraviolet spectrophotometry at 260 and 280 nm. The A_{260}/A_{280} ratios of all samples used for Northern blot analysis were between 1.6 and 2.0. RNA samples (10 μg per lane) were denatured with glyoxal and electrophoretically separated in a 1.2% agarose gel [40]. The RNA was electrophoretically transferred to a nylon membrane and UV-crosslinked. The blots were prehybridized in 50% formamide, 5 × SSPE, 5 × Denhardt's, 1 mg/ml salmon testes DNA, and 0.5% SDS and then hybridized in the same solution containing 4% dextran sulfate and one or more labeled cDNA probes. Probes were labeled with [α-^{32}P]d-cytidine triphosphate by random primer labeling using a commercially available kit from Gibco BRL. The hybridization temperature was 42°C for all probes used. The blots were washed twice in 5 × SSPE/0.5% SDS at 25°C and then twice in 0.5 × SSPE/1% SDS at 65°C. A 0.24 to 9.5 kb ladder from Gibco BRL was used for molecular size determinations. The signal was detected by phosporimaging, and the bands were quantified using ImageQuant software. When necessary, the membrane was hybridized after washing again at 100°C in water for 30 minutes.

Immunofluorescence

Immunofluorescence was performed as previously described [32]. In brief, cells on coverslips were inverted onto diluted antibodies overnight. The coverslips were subsequently incubated with biotinylated secondary antibody and then with Texas Red-avidin and bodipy-phallacidin, together. The coverslips were mounted in Gelvatol [41]. Cells were photographed using Kodak ecktachrome 400 film with an Olympus BH-2 microscope equipped for epifluorescence.

Western blot

Western blot analysis was performed as described previously [32]. To summarize, reduced proteins were transferred from gels to PVDF membranes from Millipore (Bedford, MA, USA) essentially as described by Towbin, Staehelin and Gordon [42]. α-SMA on membranes was stained using alkaline phosphatase-avidin-biotin complex (ABC) according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA, USA). Blots were developed with NBT-BCIP, BioRad (Hercules, CA, USA), prepared according to instructions.

Analysis of mesangial cell size by flow cytometry

Mesangial cells were plated in 100-mm dishes in RPMI 1640 with serum at 200,000 cells/dish or 10^6 cells/ml. Cells plated at the latter density were changed to medium without serum after four hours. Cells in medium with serum received either no addition (+serum) or 10 ng/ml TGF-β_1 (+serum, +TGF-β_1). Cells in RPMI 1640 without serum received either no addition (-serum), 10 ng/ml TGF-β_1 (−serum, +TGF-β_1), 10 ng/ml PDGF-BB (−serum, +PDGF-BB), both PDGF-BB and TGF-β_1 (−serum, +PDGF, +TGF-β_1), 10 nM thrombin (−serum, +thrombin), or 200 μM thrombin receptor-activating peptide (−serum, +SFLLRN). These agents were added on days 0, 2, and 5 after plating. On day 7 the cells were released with trypsin and resuspended in 10 ml of RPMI 1640 with FBS. The cells were placed on ice. Aliquots of the cells were counted using a Coulter Counter. The cells were centrifuged at 600 × g at 4°C for five minutes and resuspended at a concentration of 10^6 cells/ml. The cells were returned to ice and then analyzed with a Becton Dickinson Facscan fluorescence-activated cell sorter using forward light scatter mode. Results were obtained as plots of number of cells versus cell size (proportional to cell surface area) in arbitrary units. Cells not used for flow analysis were rinsed twice in PBS and extracted for Western blot analysis of α-SMA.

Preparation of images

Images prepared from blots or photomicrographs of fluorescence microscopy were printed on a Epson Stylus Color 600 printer. Original blots or ektachrome slides were scanned with either a LaCie flatbed scanner or Nikon film scanner using Adobe Photoshop software. Densitometric measurements of Western blot band densities (arbitrary units) were determined using NIH Image software version 1.59 by Wayne Rasband. All images of a particular experimental condition, blot or gel were processed identically unless stated otherwise.

RESULTS

Expression of α-smooth muscle actin in serum-deprived mesangial cells

Increased α-SMA expression has been observed to accompany mesangial cell proliferation in glomerular disease [4, 6, 9, 10]. Based on this observation, we hypothesized that serum-deprivation would arrest proliferation and cause loss of α-SMA expression in cultured mesangial cells. Human mesangial cells were plated under three conditions to test this hypothesis: in medium with serum, and in medium without serum on either serum-coated or non-coated coverslips. Figure 1 shows the effect of plating cells with or without serum on α-SMA expression after three days. The cells were fixed with paraformaldehyde and double-labeled with bodipy-phallacidin and α-SMA-specific antibody (indirect immunofluorescence; Texas Red). Phallacidin binds to filamentous actin (f-actin) of any type but not to globular actin (g-actin) [43]. Mesangial cells in serum were small and often spindle-shaped, but cells in serum-free medium were broad and polygonal. In serum, the actin filaments were thinner and less abundant (Fig. 1A) than in serum-free medium (Fig. 1 C, E). Contrary to our hypothesis, mesangial cells in RPMI 1640 with serum stained very weakly, if at all, for α-SMA by immunofluorescence, but mesangial cells in RPMI 1640 alone stained universally and intensely. Cells in serum-free medium expressed α-SMA regardless of whether they were plated onto serum-coated (Fig. 1D) or non-coated (Fig. 1F) glass coverslips.

The reversibility of the effects of serum deprivation on α-SMA expression was investigated by both Northern and Western blotting (Fig. 2). Cells were washed and plated in 100 mm culture dishes either in medium with serum or without serum. Three days after plating, cells in some of the dishes were released with trypsin, washed, and extracted for analysis of mRNA and protein. Media were changed on the remaining dishes. Some were continued in the same type of medium, either RPMI 1640 with 16.7% FBS (serum-plus) or without FBS (serum-minus), but others were changed from either serum-plus to serum-minus or from serum-minus to serum-plus. Two days later the remaining dishes were extracted. Figure 2A shows hybridization with probes for α-SMA, β-actin, GAPD and 18S ribosomal RNA. Equal amounts of RNA were analyzed. Marked differences in α-SMA mRNA levels were immediately apparent between cells in medium with or without serum. Band intensities were quantified using a phosphor-imager. Relative band intensities (summarized in Table 1) were normalized to their values in serum on day 3 (S+). β-actin, GAPD, and 18S RNA differed in other lanes by twofold or less, but α-SMA mRNA increased tenfold with serum deprivation for three days or five days. Two days after changing the medium from serum-minus to serum-plus (S− > S+), the level of α-SMA mRNA decreased from 10.6-fold to only 2.2-fold of the level in S^+ cells on day 3. Therefore, the effects on α-SMA expression of adding or removing serum could be reversed.

One dish each of cells treated as in Figure 2 was extracted for analysis of α-SMA by Western blot (Fig. 2B). Serum-plus medium decreased α-SMA protein levels as it did mRNA levels. After three days, the α-SMA band density in serum-minus cells (S−) was 5.3-fold its density in serum-plus cells (S+). The level of α-SMA protein continued to decline in serum-plus medium, and after five days (S+ > S+), was only one fifth of its level after three days (S+). The band density of α-SMA from cells serum-minus for five days (S− > S−) was 56-fold that of cells serum-plus for five days (S+ > S+). In 16 of 16 experiments, cells in medium with serum for one to three days contained markedly less α-SMA than cells in medium without serum.

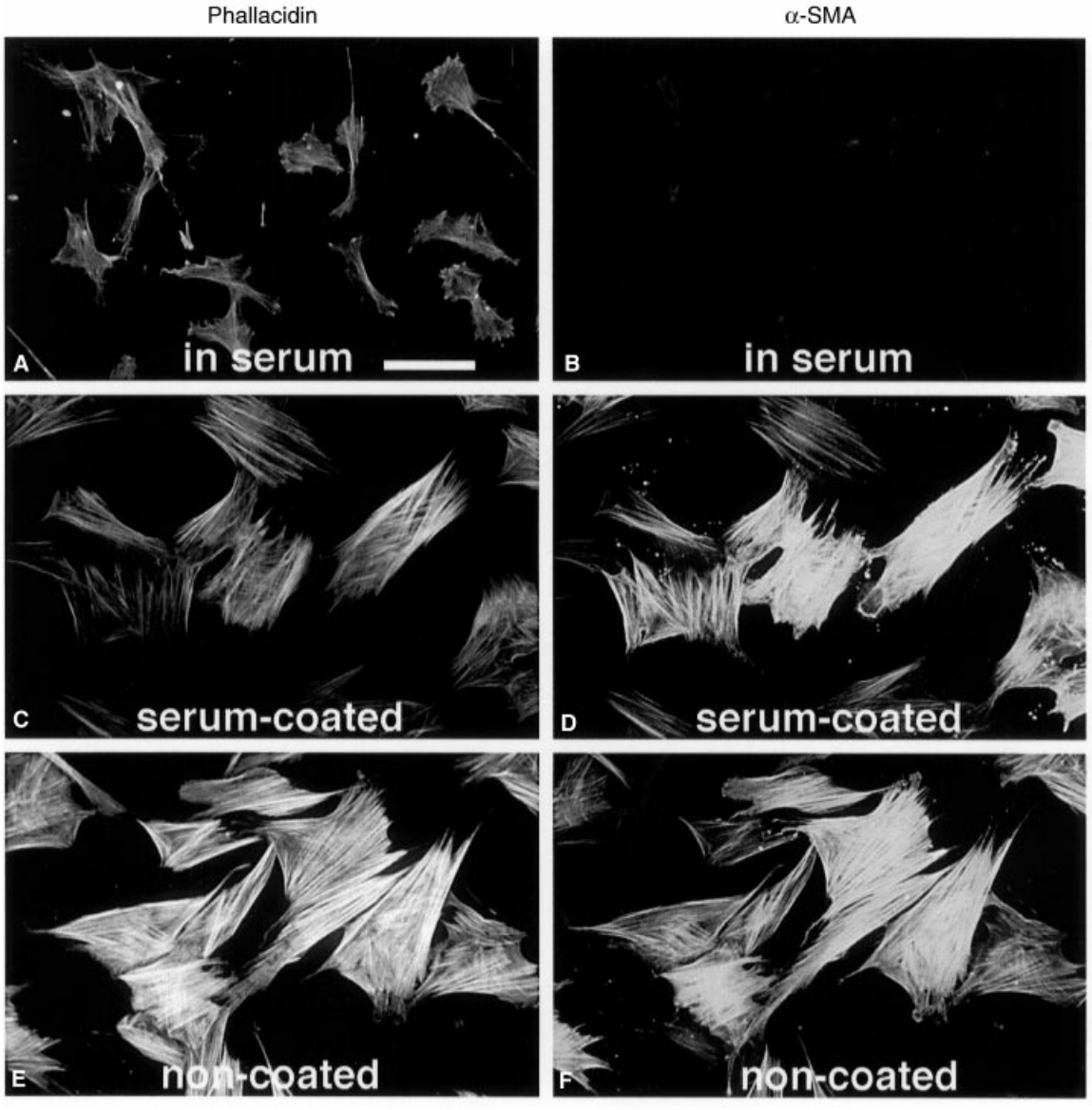

Fig. 1. Fluorescence staining of total F-actin and α-smooth muscle actin (α-SMA) in human mesangial cells. Human mesangial cells were washed and plated (40,000 cells/well) in RPMI 1640 with (*A and B*) and without (*C through F*) 16.7% serum onto non-coated (A, B, E, F) and serum-coated glass coverslips (C, D). Three days after plating, the cells were fixed and double-labeled with bodipy-phallacidin (A, C, E) for total f-actin and by indirect immunofluorescence (Texas Red) for α-SMA (B, D, F). Bar = 116 μm.

Expression of α-smooth muscle actin in serum-deprived rat and mouse mesangial cells

Many published studies of α-SMA expression in disease have involved animal models, primarily rats. Figure 3 compares expression of α-SMA by rat and mouse mesangial cells treated as in Figure 2 in serum-plus and serum-minus medium. Interestingly, expression of α-SMA in these species in the presence of serum greatly exceeded the expression of α-SMA in human mesangial cells in serum. Nonetheless, expression of α-SMA was less in the presence of serum than in serum-free medium. Similar results were obtained by immunofluorescence (not shown).

Effect of platelet derived growth factor-BB and thrombin

The abilities of two serum constituents, PDGF-BB and thrombin, to affect α-SMA expression in human mesangial cells were determined. Figure 4 shows that two days after adding thrombin the band density of α-SMA by Western blot was about 50% of control, and after adding recombinant human PDGF-BB, the level was about 40% of control. PDGF-BB decreased α-SMA expression by Western blot in 8 of 8 experiments, and thrombin decreased it in 6 of 7 experiments. The effects of these agents on α-SMA mRNA levels were also determined. RNA was extracted at 0, 4, 8, and 24 hours after the addition of either thrombin or

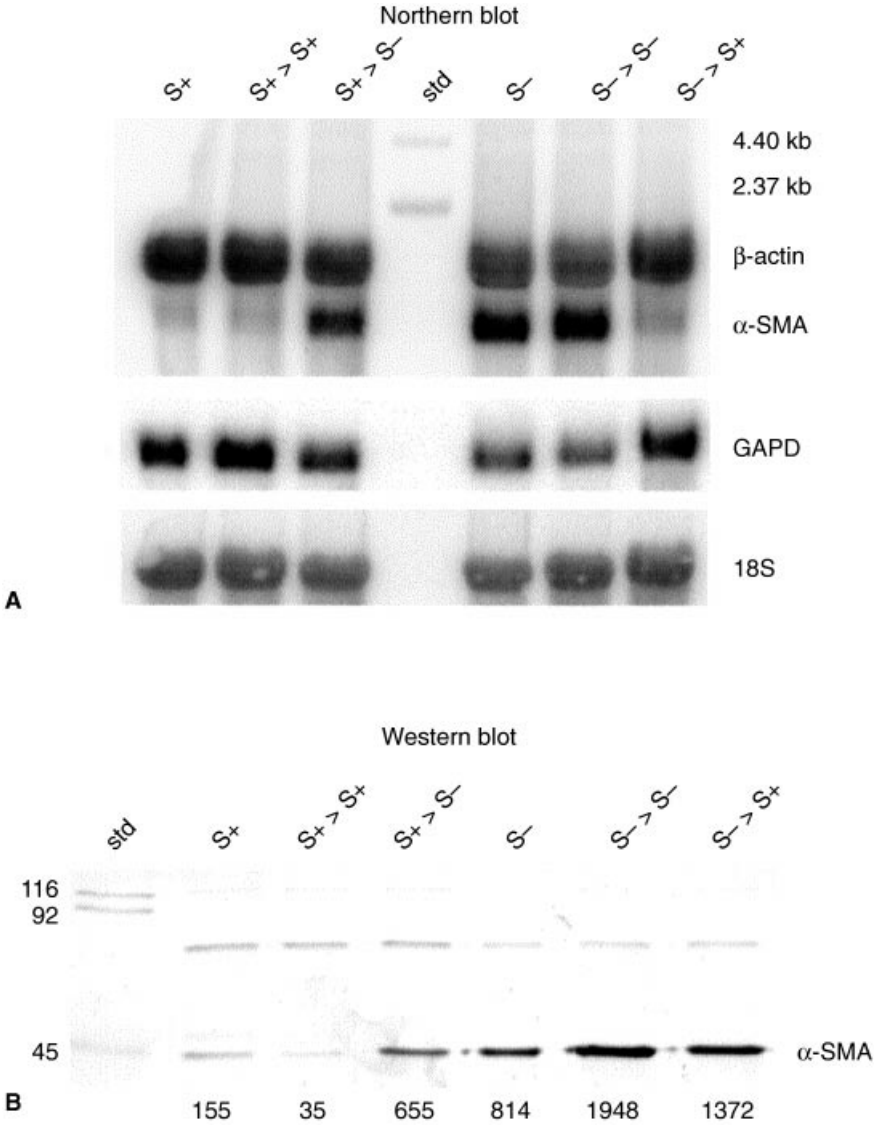

Fig. 2. Effect of serum or α-SMA mRNA in human mesangial cells by Northern and Western blots. Cells were plated either in medium with serum (S+) at 200,000 cells/100 mm dish or without serum in non-coated dishes (S−) at 10^6 cells/100 mm dish. Three days after plating RNA (5 dishes) and protein (one dish) were extracted from some dishes in each group (S+, S−). The medium was changed on the remaining dishes. Six dishes for each condition were continued in the same medium, either with serum (S+ > S+) or without serum (S− > S−). Six dishes for each were changed from medium with serum to medium without serum (S+ > S−) or from medium without serum to medium with serum (S− > S+). On day 5, RNA and protein were extracted from the remaining dishes. The RNA (10 μg/lane) was analyzed by Northern blot with cDNA probes for α-SMA and β-actin, and then stripped and reprobed for GAPD, followed by 18S ribosomal RNA (*A*). Quantified results are summarized in Table 1. One dish each of cells treated as described above was extracted and analyzed by Western blot for α-SMA (*B*). Band densities in arbitrary units determined using NIH Image Software are shown below each α-SMA band.

Table 1. Relative levels of α-smooth muscle actin (α-SMA) mRNA in mesangial cells in serum-plus (S+) and serum-minus (S−) medium determined by Northern blot (Fig. 2) normalized to their values in serum-plus cells on day 3

Day 0 Day 3 Day 5	S+ serum + extract	S+ > S+ serum + serum + extract	S+ > S− serum + serum − extract	S− serum − extract	S− > S− serum − serum − extract	S− > S+ serum − serum + extract
β-actin	1.0	1.0	0.7	0.6	0.5	0.9
α-SMA	1.0	1.3	7.0	10.6	10.7	2.3
GAPD	1.0	1.5	0.8	0.6	0.5	1.2
18S	1.0	1.0	0.9	0.9	1.0	1.0

PDGF-BB and analyzed by Northern blot (Fig. 5). Band intensities were quantified using a phosphor-imager. Relative band intensities (summarized in Table 2) were normalized to the values at time 0. The effects of thrombin and PDGF-BB on α-SMA mRNA followed a similar time course. The α-SMA mRNA level declined slightly in the first eight hours, and after 24 hours was about one-fifth of control (0 hr) in both cases.

Effect of transforming growth factor-β_1

Transforming growth factor-β has been reported to stimulate α-SMA expression in smooth muscle cells [44]

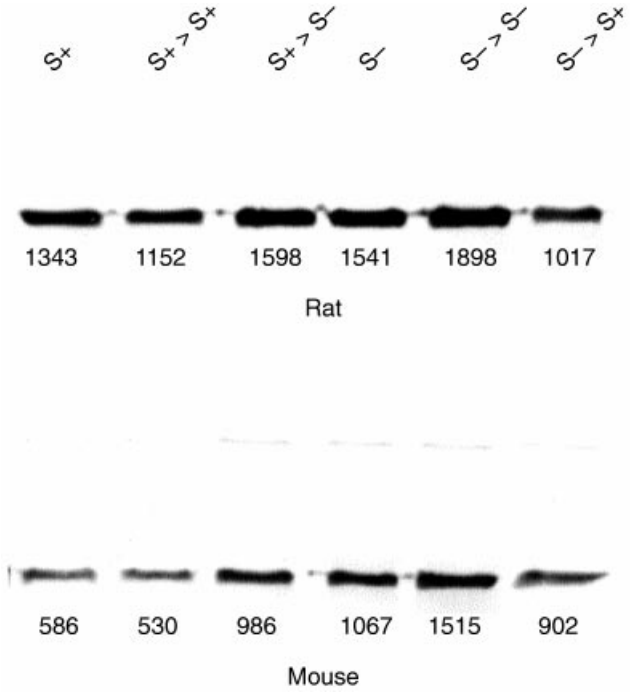

Fig. 3. Effect of serum on α-SMA protein in rat and mouse mesangial cells by Western blot. Rat and mouse mesangial cells were cultured and the cells were treated as described in Figure 2. Lane numbers and legends are the same as Figure 2.

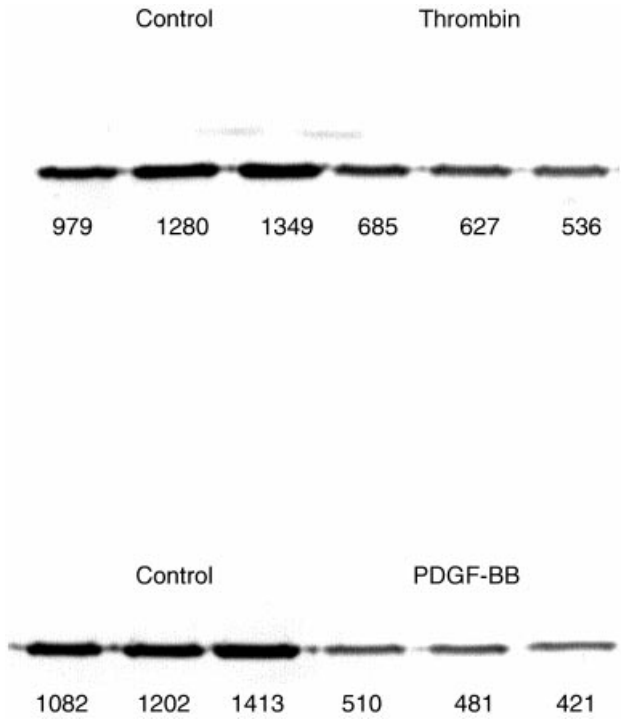

Fig. 4. Western blot analysis of the effects of thrombin and PDGF-BB on α-SMA. Washed cells (200,000 cells/well of 6-well plates) were plated in RPMI 1640 without serum. Five days after plating, the cells were changed to fresh RPMI 1640 alone (control) or containing either 5 nM thrombin or 10 ng/ml PDGF-BB. After seven days the cells were extracted in electrophoresis sample buffer and analyzed by Western blot (2 μg/lane). Extracts from three wells per condition are shown.

and fibroblasts [45]. The hypothesis that TGF-β1 increases α-SMA expression in human mesangial cells was tested. Cells in either serum-plus or serum-minus medium for three days were treated with 10 ng/ml TGF-β1 for two days. Next the cells were extracted and analyzed by Northern blot for β-actin, α-SMA, GAPD, and 18S ribosomal RNA (Fig. 6A). Analysis by phosphor imaging, summarized in Table 3, revealed that TGF-β1 caused about a threefold (range 2.4- to 4.0-fold) increase in the α-SMA mRNA level in the presence of serum, but had little or no effect on the α-SMA mRNA level in serum-minus cells. Although the initial intent had been to normalize the data to GAPD or 18S RNA, the levels of these RNA species were noted to decrease with serum deprivation to varying degrees in different experiments. In this experiment, with samples prepared in triplicate, serum-minus cells contained about one-fifth as much GAPD mRNA and about one-third as much 18S RNA as serum-plus cells. The cause for the observed variability in the effects of serum deprivation on GAPD and 18S RNA is not known. However, within either serum-plus or serum-minus conditions, none of the agents studied (thrombin, PDGF, and TGF-β1) appeared to affect GAPD or 18S levels. Nonetheless, in response to serum deprivation, these RNA species decrease while α-SMA mRNA increases. Consequently, normalization of the data to either of theses species would indicate even greater increases (about 30- to 60-fold) in α-SMA mRNA levels due to serum deprivation.

Extracts of cells from the same experiment were analyzed by Western blot for α-SMA (Fig. 6B). α-SMA protein, like its mRNA, increased in cells treated with TGF-β1 in medium with serum, about two-fold, but did not increase in cells treated in medium without serum.

Regulation of cell size and α-smooth muscle actin expression

Transforming growth factor-β stimulates α-SMA expression in smooth muscle cells [44] and fibroblasts [45], and causes hypertrophy of rat mesangial cells [46]. Human mesangial cells in RPMI 1640 alone appeared larger than mesangial cells in serum (Fig. 1). These observations suggested that the serum-free mesangial cells underwent hypertrophy. The possibility that agents that alter mesangial cell α-SMA content also alter cell size was investigated by analyzing mesangial cells with a Becton Dickinson Facscan fluorescence-activated cell sorter using forward light scatter mode. The cells were treated three times over the course of a week with fresh medium containing either no additional agent or 10 ng/ml TGF-β1, 10 ng/ml PDGF-BB, 10 nM thrombin or 200 μM SFLLRN peptide, alone or in combination. At the end of one week, the cells were harvested by trypsinization and analyzed. Figure 7 shows graphically the distribution of mesangial cell sizes under

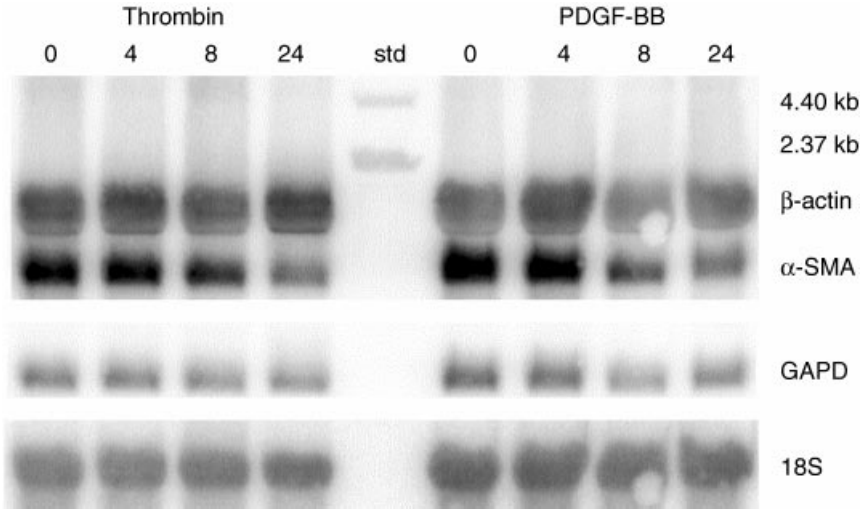

Fig. 5. Expression of α-SMA mRNA in mesangial cells in serum-free medium containing thrombin or PDGF. Washed cells (10^6 cell/100 mm dish) were plated without serum. Three days after plating, the cells were treated with either 10 nM thrombin or 10 ng/ml PDGF-BB. RNA was extracted from the cells after 0 hours (0), four hours (4), eight hours (8), and 24 hours (24). The RNA (10 μg/lane) was analyzed by Northern blot with cDNA probes for α-SMA and β-actin, and then stripped and reprobed for GAPD, followed by 18S ribosomal RNA. Quantified results are summarized in Table 2.

Table 2. Relative levels of α-SMA mRNA in mesangial cells treated with thrombin or PDGF-BB determined by Northern blot (Fig. 5) normalized to their values at time 0

	Thrombin *10 nM*				PDGF-BB *10 ng/ml*			
Time	0 hr	4 hr	8 hr	24 hr	0 hr	4 hr	8 hr	24 hr
β-actin	1.0	0.8	1.6	0.5	1.0	1.2	1.1	0.8
α-SMA	1.0	0.9	0.6	0.2	1.0	0.9	0.3	0.2
GAPD	1.0	1.0	0.7	0.7	1.0	0.9	0.4	0.5
18S	1.0	1.0	1.1	1.2	1.0	0.9	0.8	0.9

each condition. Cells from the same experiment were extracted and analyzed by Western blot for α-SMA (Fig. 8). Regardless of the agent added, cells in serum-minus medium contained more α-SMA than cells in serum-plus medium. To avoid saturated staining (as in Fig. 6), less protein extracted from cells treated under serum-minus conditions (0.2 μg, panels/lanes C-H) than serum-plus conditions (1 μg, panels/lanes A, B) was analyzed by Western blot. In each case, cellular enlargement was accompanied by increased α-SMA expression. Cells plated without serum (panel/lane C) were larger and contained more α-SMA than cells plated with serum (panel/lane A). Cells treated with TGF-β_1 in serum-plus medium (panel/lane B) were larger and contained more α-SMA than cells in serum-plus medium alone (panel/lane A). The addition of TGF-β_1 to serum-minus cells (panel/lane D) had no effect on either cell size or α-SMA content compared to serum-minus cells alone (panel/lane C). PDGF-BB prevented enlargement of mesangial cells in serum free medium (Fig. 7E) and antagonized α-SMA expression (Fig. 8, lane E). Cells treated with both PDGF-BB and TGF-β_1 (panel/lane F) contained an amount of α-SMA intermediate between that of cells treated with either PDGF-BB (panel/lane E) or TGF-β_1 (panel/lane D) alone. Thrombin (panel/lane G), like PDGF-BB, reduced mesangial cell size and α-SMA content compared to serum-minus medium alone (panel/lane C). Thrombin receptor-activating peptide (SFLLRN) also decreased the size and actin content of mesangial cells (panel/lane H), but to a lesser extent than thrombin.

The effect of TGF-β_1 and PDGF-BB in combination was further investigated. Three separate experiments were done following the same experimental protocol as above, but varying the concentration of PDGF-BB. Both forward light scattering and α-SMA expression were analyzed. The results of each experiment were normalized to the values for serum-minus cells (defined as 100%) and ranged from 80% to 127%. Figure 9 shows average results from the three experiments. Although absolute values varied between experiments, the trends shown were present in each experiment. Cells in serum-plus medium were smallest (88%) and contained the least amount of α-SMA. Cells treated with TGF-β_1 in the presence of serum were the largest, and on average, contained four times as much α-SMA. Cells in serum-minus medium contained the most α-SMA, about 20 times as much as serum-plus cells; the addition of TGF-β_1 to serum-minus cells had little effect on cell size or α-SMA expression. Addition of PDGF-BB to serum-minus medium caused a dose-dependent decrease in cell size and α-SMA expression that was offset by addition of TGF-β_1. The effect of TGF-β_1 appeared greater at lower PDGF-BB concentrations.

DISCUSSION

Our studies show that expression of α-SMA mRNA and protein in cultured mesangial cells correlates better with hypertrophy rather than proliferation. Human mesangial cell mitogens (serum, PDGF-BB [33] and thrombin [33]) inhibited α-SMA expression and antagonized hypertropy. Conditions that inhibited proliferation (TGF-β_1 [34] or serum-free medium) increased α-SMA expression and caused hypertrophy.

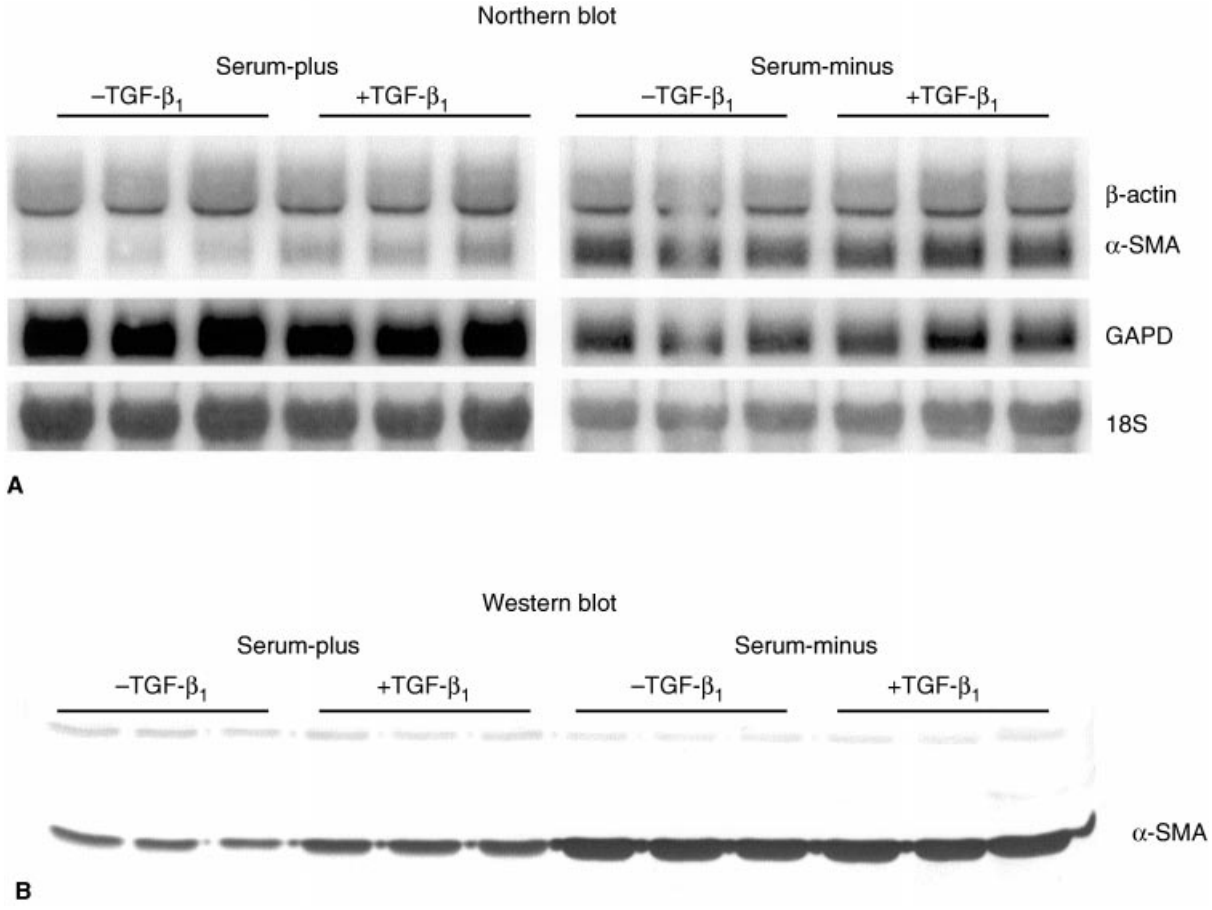

Fig. 6. Effect of TGF-B_1 on α-SMA in human mesangial cells by Northern and Western blots. Mesangial cells were plated at a density of either 400,000/100 mm dish (6 dishes) in RPMI 1640 plus serum or at 2×10^6/100 mm dish (6 dishes) in RPMI 1640 alone. After three days, the cells were treated with fresh medium (serum-minus or serum-plus) with or without 10 ng/ml TGF-B_1 for two days. The cells were detached with trypsin and cells from duplicate dishes were pooled and extracted, giving triplicate samples for each condition. The RNA (10 μg/lane) was analyzed by Northern blot with cDNA probes for α-SMA and β-actin, and then stripped and reprobed for GAPD, followed by 18S ribosomal RNA. Quantified results are summarized in Table 3. An aliquot of cells from each sample was extracted in electrophoresis sample buffer and 5 μg protein/lane was analyzed by Western blot (*B*).

Table 3. Relative levels of α-SMA mRNA in mesangial cells treated with TGF-β1 determined by Northern blot (Fig. 6) in triplicate normalized to their average values in serum-plus medium without TGF-β1

TGF-β1	Serum-plus						Serum-minus					
	−	−	−	+	+	+	−	−	−	+	+	+
β-actin	0.9	0.8	1.2	1.0	0.9	1.2	0.8	0.5	0.8	0.7	0.9	0.9
αSMA	1.1	0.7	1.2	2.9	2.4	4.0	12.8	7.9	9.4	9.9	13.6	12.4
GAPD	1.0	0.8	1.1	0.7	0.6	0.9	0.2	0.1	0.2	0.2	0.3	0.2
18S	1.1	0.9	1.0	0.8	0.7	0.9	0.4	0.3	0.4	0.4	0.5	0.6

The finding that serum deprivation increases α-SMA protein and mRNA levels was surprising given the results of studies using reporter constructs that contain α-SMA promoter regions [12, 31]. The rat promoter contains two $CC(A/T)_6GG$ sequences (CArG box elements) responsible for binding of serum response factor. Thus, one would expect that α-SMA mRNA transcription would be increased in medium containing serum compared to medium without serum. Nevertheless, the effect of serum deprivation on α-SMA mRNA and protein is not described in either of these papers. One possible explanation for our unexpected finding is that although serum increases α-SMA transcription, it increases α-SMA mRNA degradation to a greater extent. Thus, serum deprivation may increase α-SMA mRNA and protein levels even though it decreases α-SMA transcription by a compensatory increase in α-SMA message stability. This is currently under investigation.

Conversion of plasma to serum endows it with its mitogenic properties [21, 22]. The factors are probably diverse; however, PDGF isoforms have probably been the best studied. PDGF activity in serum varies widely between species. In one study, human serum contained 13.3 ± 4.5 ng/ml total PDGF activity (6.25 ± 2.2% BB isoform) while FBS contained 0.93 ± 0.3 ng/ml (82 ± 12% BB isoform) [47]. The mitogenic potency of human and bovine sera were similar despite these differences, suggesting that other substances were as important as PDGF in giving serum its

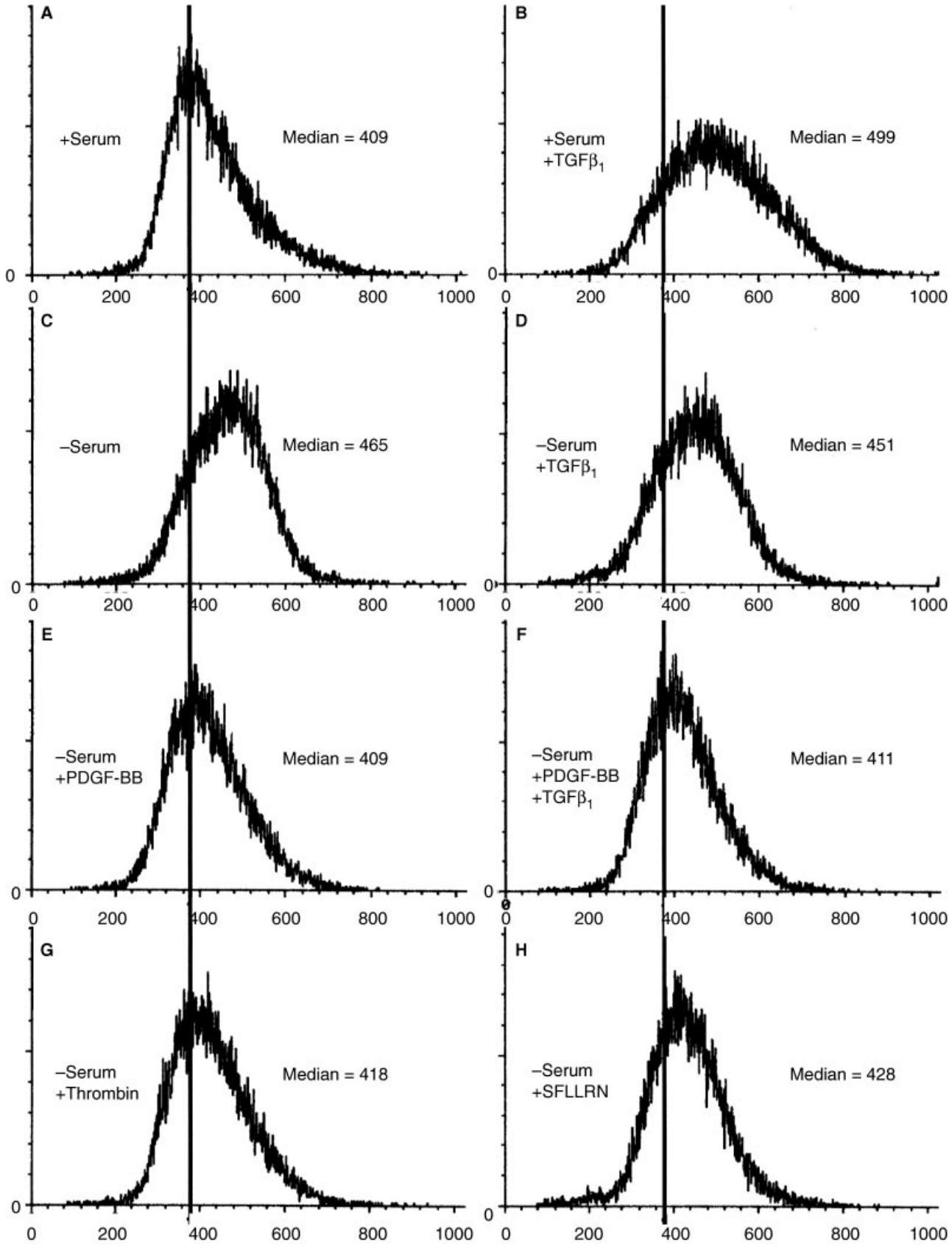

Fig. 7. Forward light scatter flow cytometry of human mesangial cells treated with TGFβ_1, PDGF-BB, thrombin, and/or SFLLRN. Cells were in RPMI 1640 with (*A* and *B*) or without serum (*C* through *H*) containing no addition (*A*), 10 ng/ml TGFβ_1, (*B*, *D*, *and F*), 10 ng/ml PDGF-BB (*E and F*), 10 nM thrombin (*G*) and/or 200 μM SFLLRN-peptide (*H*). Media containing the appropriate agent(s) were changed twice and the cells were released with trypsin and analyzed after one week. The vertical line provides a reference for comparison to the position of the peak in cells grown in medium with serum (A). The number of cells counted (y-axis; 15 cells/division) is plotted against cell size (x-axis; arbitrary units). The median cell size (arbitrary units) is noted for each condition.

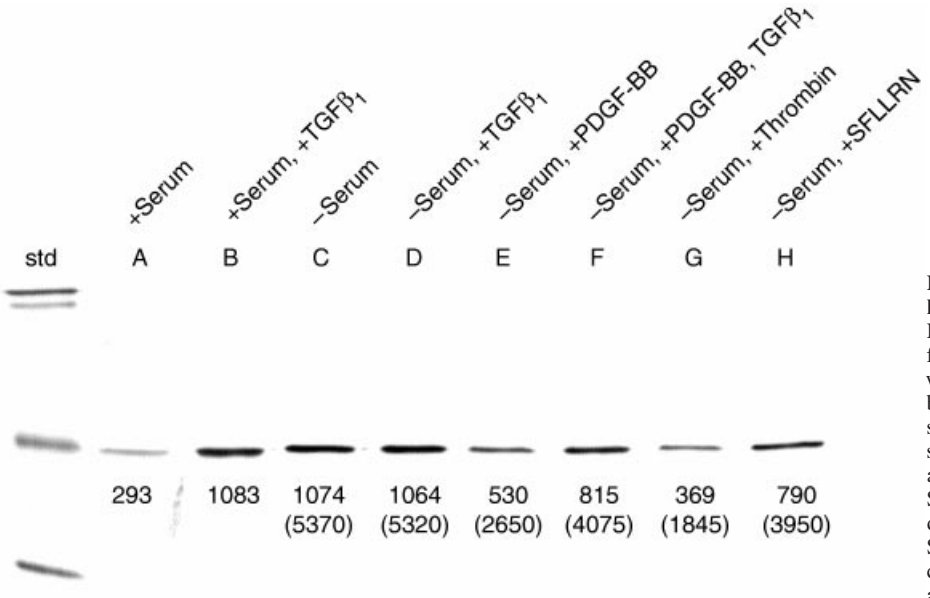

Fig. 8. Western blot analysis of α-SMA in human mesangial cells treated with TGF-β_1, PDGF-BB, thrombin, and/or SFLLRN. Cells from the experiment described in Figure 7 were washed and extracted in electrophoresis sample buffer. More sample was analyzed for cells in serum-plus medium (1 μg), than for cells in serum-minus medium (0.2 μg) in order to allow a more accurate relative quantitation of α-SMA. Values below bands show the results of densitometric analysis using the NIH Image Software. Values in parentheses have been corrected for differences in the total protein analyzed.

mitogenic properties. These unidentified substances could also affect α-SMA expression and mesangial cell size.

Thrombin is present in serum. Whether thrombin contributes significantly to the mitogenic activity of serum is unclear because of the abundance of protease inhibitors in serum. However, the active concentration of thrombin in fresh 10% FBS was 10 ng/ml (0.3 nM) and after three days at 37°C, it was 28 ng/ml (0.8 nM) in one investigation [48]. Our experiments were conducted with 5 to 10 nM thrombin. PDGF-BB and/or thrombin may contribute to inhibition of α-SMA expression by serum, but inhibition of α-SMA expression by serum is probably the sum of diverse components.

Thrombin causes mesangial cells to secrete PDGF [33], and thus, inhibition of α-SMA expression by thrombin might have been the result of autocrine stimulation by PDGF. However, neutralizing-antibody to PDGF-BB (also PDGF-AB) did not block the effect of thrombin on α-SMA expression at concentrations in excess of that necessary to block 10 ng/ml PDGF-BB (not shown). Hence, the effect of thrombin on α-SMA expression seems to be independent of PDGF-BB secretion.

If a similar relationship between hypertrophy and α-SMA expression in mesangial cells exists *in vivo*, then expression of α-SMA in conditions where there is little proliferation can be explained. For example, angiotensin II caused increased expression of α-SMA in the mesangial cells of treated rats without causing mesangial cell proliferation [8]. Increased expression of α-SMA has been observed in human [11] and experimental diabetic glomerular injury [49]. The authors of the latter study [49] suggested that increased expression of contractile proteins in such diseases might serve as a marker for glomerular hypertrophy. Thus, expression of α-SMA under these *in vivo* conditions may indicate mesangial cell hypertrophy.

Cells may undergo hyperplasia and hypertrophy simultaneously. TGF-β_1 caused mesangial cell hypertrophy and increased α-SMA expression in serum-plus medium without fully antagonizing the hyperplastic response of the cells to serum (Fig. 8). After one week, the dish with serum-plus medium alone contained 16-times as many cells as plated, while the dish with serum-plus medium and 10 ng/ml TGF-β_1 contained 10 times as many cells as plated. Therefore, cells in serum-plus medium with TGF-β_1 underwent hyperplasia (albeit at a slower rate) and hypertrophy simultaneously. Increased α-SMA expression in proliferative diseases may reflect concurrent hypertrophy and hyperplasia *in vivo*. TGF-β production in diseases could be responsible. TGF-β is known to stimulate α-SMA synthesis in smooth muscle cells [44] and fibroblasts [45], and it causes hypertrophy of rat mesangial cells [46].

Cells in serum-plus medium alone were smallest, but cells in serum-plus medium with TGF-β_1 were largest (even larger than serum-minus cells with TGF-β_1). This suggests that serum contains a factor or factors that have a synergistic effect on TGF-β_1-induced hypertrophy. TGF-β_1 increased expression of α-SMA in cells in serum-plus medium but not serum-minus medium. However, the latter contained more α-SMA, with or without TGF-β_1, than cells in serum-plus medium with TGF-β_1. These differences in the response to TGF-β_1 of cells in medium with or without serum may be due to the presence of a complex mixture of factors in serum, including some that inhibit α-SMA expression. The observation that no clear response to TGF-β_1 was observed in medium without serum could be due to

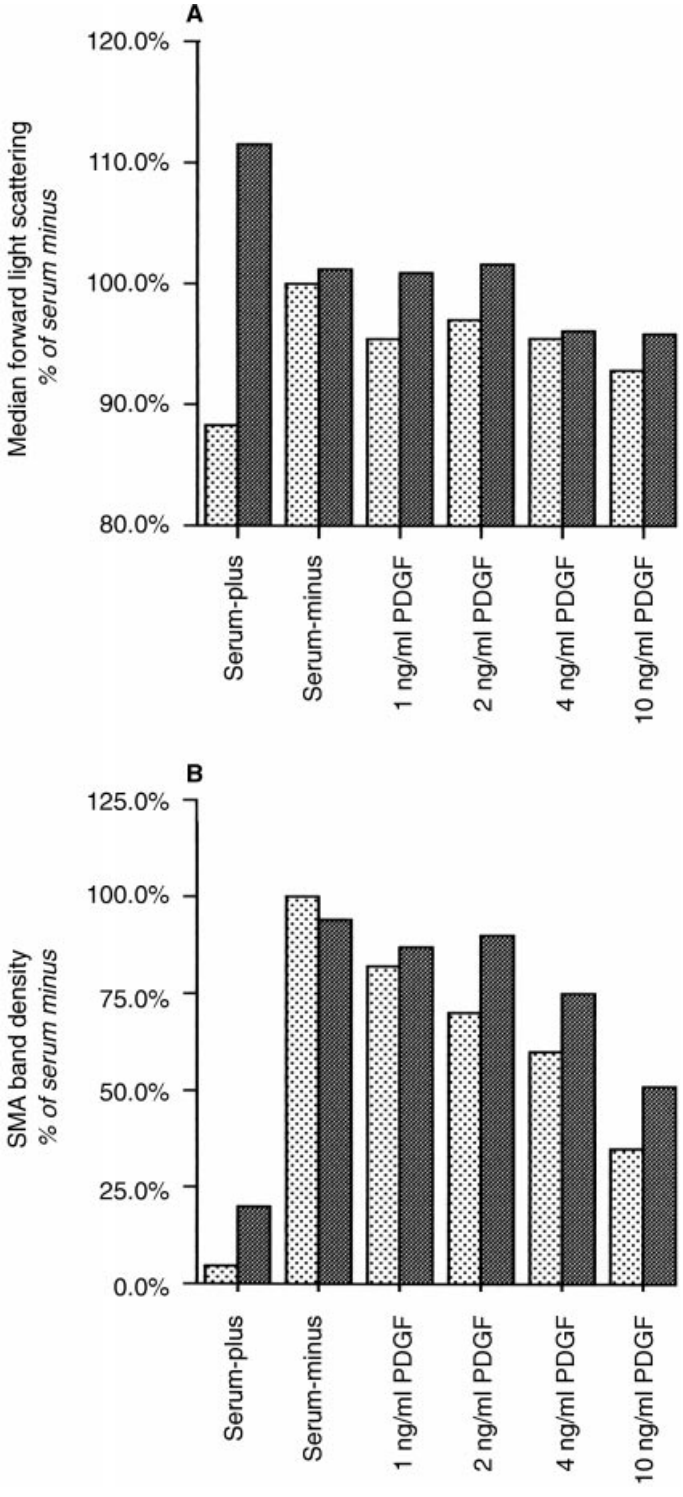

Fig. 9. Forward light scatter flow cytometry and α-SMA expression in mesangial cells treated with TGF-β_1 and various concentrations of PDGF-BB. Cells were treated with 10 ng/ml TGF-β_1 in RPMI 1640 with or without 16.7% FBS (serum-plus and serum-minus, respectively) or in RPMI 1640 containing 1, 2, 4, or 10 ng/ml PDGF-BB. The same protocol was followed as in Figure 8. The results are the averages of three experiments. Median forward light scattering and α-SMA band density were normalized to the values of serum-minus cells (100%) before averaging. Symbols are: (⊡) without TGF-β_1; (▨) with TGF-β_1.

absence of serum factors that enhance the responsiveness of cells to TGF-β_1. From published observations [47], the concentration of PDGF-BB in 16.7% serum can be estimated at 1.3 ng/ml. Although the cells were not responsive to TGF-β1 when serum deprived, TGF-β1 nonetheless antagonized the effect of PDGF-BB (Figs. 7, 8 and 9). This suggests that PDGF-BB may partially restore TGF-β responsiveness to the serum-deprived mesangial cells, perhaps through affecting TGF-β1 receptor expression. Clearly, diverse substances in serum may alone or in combination be responsible for the effect of TGF-β_1 and serum together on cell size and α-SMA expression.

Increased expression of α-SMA has been observed in other cell types undergoing hypertrophy. Hypertrophy of vascular smooth muscle cells treated in culture with angiotensin II or arginine vasopressin is accompanied by a selective increase in α-SMA expression [50]. During cardiac hypertrophy in response to increased load caused by aortic coarctation there is reactivation of α-SMA expression [51]. Hence, increased α-SMA expression may be a general manifestation of hypertrophy in cells of mesenchymal origin.

In summary, human mesangial cells deprived of serum enlarge and develop dense bundles of stress fibers rich in α-SMA. This process, hypertrophy, was antagonized reversibly by serum, thrombin, and PDGF-BB. TGF-β_1 caused hypertrophy and increased α-SMA expression in the presence of serum. Diverse factors probably act on mesangial cells cultured in the presence of serum or *in vivo* in the setting of inflammation or sclerosis to regulate the level of α-SMA expression, but enhanced expression of α-SMA is primarily reflective of conditions favoring hypertrophy rather than hyperplasia.

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Reprint requests to William F. Glass II, Department of Pathology, Eastern Virginia Medical School, P. O. Box 1980, 700 Olney Road, Norfolk, Virginia 23501-1980, USA.
E-mail: wfg@evms.edu.

APPENDIX

Abbreviations used in this article are: α-SMA, α-smooth muscle actin; f-actin, filamentous actin; FBS, fetal bovine serum; g-actin, globular actin; GAPD, glyceraldehyde phosphate-3-dehydrogenase; PDGF, platelet derived growth factor; S−, serum-minus cells; S+, serum-plus cells; SFLLRN, thrombin receptor-activating peptide; TGF-β_1, transforming growth factor-β_1.

REFERENCES

1. Holthofer H, Sainio K, Miettinen A: The glomerular mesangium: Studies of its developmental origin and markers in vivo and in vitro. *APMIS* 103:354–366, 1995
2. Alpers CE, Seifert RA, Hudkins KL, Johnson RJ, Bowen-Pope DF: Developmental patterns of PDGF B-chain, PDGF-receptor, and alpha-actin expression in human glomerulogenesis. *Kidney Int* 42:390–399, 1992
3. Carey AV, Carey RM, Gomez RA: Expression of alpha-smooth muscle actin in the developing kidney vasculature. *Hypertension* 19:II168–II175, 1992
4. Roy-Chaudhury P, Wu B, McDonald S, Haites NE, Simpson JG, Power DA: Phenotypic analysis of the glomerular and periglomerular mononuclear cell infiltrates in the Thy 1.1 model of glomerulonephritis. *Lab Invest* 72:524–531, 1995
5. Romano LA, Ferder L, Inserra F, Ercole L, Gomez RA: Intraglomerular expression of alpha-smooth muscle actin in aging mice. *Hypertension* 23:889–893, 1994
6. Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen-Pope DF, Couser WG, Johnson RJ: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41:297–309, 1992
7. Floege J, Johnson RJ, Couser WG: Mesangial cells in the pathogenesis of progressive glomerular disease in animal models. (Review) *Clin Invest* 70:857–864, 1992
8. Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM: Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19:464–474, 1992
9. Johnson RJ, Iida H, Alpers CE, Majesky MW, Schwartz SM, Pritzi P, Gordon K, Gown AM: Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J Clin Invest* 87:847–858, 1991
10. Macpherson BR, Leslie KO, Lizaso KV, Schwarz JE: Contractile cells of the kidney in primary glomerular disorders: An immunohistochemical study using an anti-alpha-smooth muscle actin monoclonal antibody. *Hum Pathol* 24:710–716, 1993
11. Alpers CE, Hudkins KL, Gown AM, Johnson RJ: Enhanced expression of "muscle-specific" actin in glomerulonephritis. *Kidney Int* 41:1134–1142, 1992
12. Simonson MS, Walsh K, Kumar CC, Bushel P, Herman WH: Two proximal CArG elements regulate SM alpha-actin promoter, a genetic marker of activated phenotype of mesangial cells. *Am J Physiol* 268:F760–F769, 1995
13. Alpers CE, Hudkins KL, Ferguson M, Johnson RJ, Schatteman GC, Bothwell M: Nerve growth factor receptor expression in fetal, mature, and diseased human kidneys. *Lab Invest* 69:703–713, 1993
14. Hugo C, Pichler R, Gordon K, Schmidt R, Amieva M, Couser WG, Furthmayr H, Johnson RJ: The cytoskeletal linking proteins, moesin and radixin, are upregulated by platelet-derived growth factor, but not basic fibroblast growth factor in experimental mesangial proliferative glomerulonephritis. *J Clin Invest* 97: 2499–2508, 1996
15. Floege J, Eng E, Young BA, Alpers CE, Barrett TB, Bowen-Pope DF, Johnson RJ: Infusion of platelet-derived growth factor or basic fibroblast growth factor induces selective glomerular mesangial cell proliferation and matrix accumulation in rats. *J Clin Invest* 92:2952–2962, 1993
16. Groma V, Marcussen N, Olsen S: A quantitiative immunohistochemical study of the expression of mesangial alpha—smooth muscle actin and the proliferative marker KI-67 in glomerulonephritis in man. *Virchows Arch* 431:345–350, 1997
17. Hayman EG, Engvall E, A'Hearn E, Barnes D, Pierschbacher M, Ruoslahti E: Cell attachment on replicas of SDS polyacrylamide gels reveals two adhesive plasma proteins. *J Cell Biol* 95:20–23, 1982
18. Kleinman HK, Luckenbill-Edds L, Cannon FW, Sephel GC: Use of extracellular matrix components for cell culture. (Review) *Anal Biochem* 166:1–13, 1987
19. Burridge K, Fath K: Focal contacts: Transmembrane links between the extracellular matrix and the cytoskeleton. (Review) *Bioessays* 10:104–108, 1989
20. Balk SD: Calcium as a regulator of the proliferation of normal, but not of transformed, chicken fibroblasts in a plasma-containing medium. *Proc Natl Acad Sci USA* 68:271–275, 1971
21. Ross R, Glomset J, Kariya B, Harker L: A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci USA* 71:1207–1210, 1974
22. Kohler N, Lipton A: Platelets as a source of fibroblast growth-promoting activity. *Exp Cell Res* 87:297–301, 1974
23. Poggi A, Vicenzi E, Cioce V, Wasteson A: Platelet contribution to cancer cell growth and migration: The role of platelet growth factors. (Review) *Haemostasis* 18:18–28, 1988
24. Gee AP, Minta JO: Migration of porcine endothelial and smooth muscle cells in response to platelet-associated factors. *Am J Hematol* 17:29–38, 1984
25. Mordan LJ: Induction by growth factors from platelets of the focus-forming transformed phenotype in carcinogen-treated C3H/10T1/2 fibroblasts. *Carcinogenesis* 9:1129–1134, 1988
26. Paul D, Piasecki A: Rat platelets contain growth factor(s) distinct from PDGF which stimulate DNA synthesis in primary adult rat hepatocyte cultures. *Exp Cell Res* 154:95–100, 1984
27. Russell WE, McGowan JA, Bucher NL: Biological properties of a hepatocyte growth factor from rat platelets. *J Cell Physiol* 119:193–197, 1984
28. Rutherford RB, Ross R: Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. *J Cell Biol* 69:196–203, 1976
29. Couser WG, Johnson RJ: Mechanisms of progressive renal disease in glomerulonephritis. (Review) *Am J Kidney Dis* 23:193–198, 1994
30. Elger M, Drenckhahn D, Nobiling R, Mundel P, Kriz W: Cultured rat mesangial cells contain smooth muscle alpha-actin not found in vivo. *Am J Pathol* 142:497–509, 1993
31. Kitamura M, Ishikawa Y: Three-dimensional matrix primes mesangial cells to down-regulation of alpha-smooth muscle actin via deactivation of CarG box elements. *Kidney Int* 53:690–697, 1998
32. Glass WF, Teng PR, Haney LB: Extracellular matrix distribution and hillock formation in human mesangial cells in culture without serum. *J Am Soc Nephrol* 7:2230–2243, 1996
33. Shultz PJ, Knauss TC, Mene P, Abboud HE: Mitogenic signals for thrombin in mesangial cells: Regulation of phospholipase C and PDGF genes. *Am J Physiol* 257:F366–F374, 1989
34. Jaffer F, Saunders C, Shultz P, Throckmorton D, Weinshell E, Abboud HE: Regulation of mesangial cell growth by polypeptide mitogens. Inhibitory role of transforming growth factor beta. *Am J Pathol* 135:261–269, 1989
35. Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF, Kerlavage AR, McCombie WR, Venter JC: Complementary DNA sequencing: Expressed sequence tags and human genome project. *Science* 252:1651–1656, 1991
36. Tso JY, Sun XH, Kao TH, Reece KS, Wu R: Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: Genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* 13:2485–2502, 1985
37. Finnegan MC, Hammond DW, Hancock BW, Goyns MH: Activation of MYCN in a case of non-Hodgkin's lymphoma. *Leukemia & Lymphoma* 18:511–514, 1995
38. Hembrough TA, Vasudevan J, Allietta MM, Glass WF, Gonias SL: A cytokeratin 8-like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes. HepG2 cells and breast carcinoma cell lines. *J Cell Sci* 108:1071–1082, 1995
39. Pankewycz OG, Guan JX, Bolton WK, Gomez A, Benedict JF: Renal TGF-beta regulation in spontaneously diabetic NOD mice with correlations in mesangial cells. *Kidney Int* 46:748–758, 1994
40. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual* (2nd ed). Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989
41. Harlow E, Lane D: *Antibodies: A Laboratory Manual*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1988
42. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of

proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354, 1979
43. Faulstich H, Zobeley S, Rinnerthaler G, Small JV: Fluorescent phallotoxins as probes for filamentous actin. (Review) *J Muscle Res Cell Motil* 9:370–383, 1988
44. Orlandi A, Ropraz P, Gabbiani G: Proliferative activity and alpha-smooth muscle actin expression in cultured rat aortic smooth muscle cells are differently modulated by transforming growth factor-beta 1 and heparin. *Exp Cell Res* 214:528–536, 1994
45. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G: Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103–111, 1993
46. Choi ME, Kim EG, Huang Q, Ballermann BJ: Rat mesangial cell hypertrophy in response to transforming growth factor-beta 1. *Kidney Int* 44:948–958, 1993
47. Bowen-Pope DF, Hart CE, Seifert RA: Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which bind to different classes of PDGF receptor. *J Biol Chem* 264:2502–2508, 1989
48. Gurwitz D, Cunningham DD: Thrombin modulates and reverses neuroblastoma neurite outgrowth. *Proc Natl Acad Sci USA* 85:3440–3444, 1988
49. Makino H, Kashihara N, Sugiyama H, Kanao K, Sekikawa T, Okamoto K, Maeshima Y, Ota Z, Nagai R: Phenotypic modulation of the mesangium reflected by contractile proteins in diabetes. *Diabetes* 45:488–495, 1996
50. Turla MB, Thompson MM, Corjay MH, Owens GK: Mechanisms of angiotensin II- and arginine vasopressin-induced increases in protein synthesis and content in cultured rat aortic smooth muscle cells. Evidence for selective increases in smooth muscle isoactin expression. *Circ Res* 68:288–299, 1991
51. Black FM, Packer SE, Parker TG, Michael LH, Roberts R, Schwartz RJ, Schneider MD: The vascular smooth muscle alpha-actin gene is reactivated during cardiac hypertrophy provoked by load. *J Clin Invest* 88:1581–1588, 1991