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Kidney International, Vol. 56 (1999), pp. 1455–1467

Transforming growth factor-β regulates tubular epithelial-myofibroblast transdifferentiation *in vitro*

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Transforming growth factor-β regulates tubular epithelial-myofibroblast transdifferentiation *in vitro*.

Background. We recently found evidence of tubular epithelial-myofibroblast transdifferentiation (TEMT) during the development of tubulointerstitial fibrosis in the rat remnant kidney. This study investigated the mechanisms that induce TEMT *in vitro*.

Methods. The normal rat kidney tubular epithelial cell line (NRK52E) was cultured for six days on plastic or collagen type I-coated plates in the presence or absence of recombinant transforming growth factor-β1 (TGF-β1). Transdifferentiation of tubular cells into myofibroblasts was assessed by electron microscopy and by expression of α-smooth muscle actin (α-SMA) and E-cadherin.

Results. NRK52E cells cultured on plastic or collagen-coated plates showed a classic cobblestone morphology. Culture in 1 ng/ml TGF-β caused only very minor changes in morphology, but culture in 10 or 50 ng/ml TGF-β1 caused profound changes. This involved hypertrophy, a loss of apical-basal polarity and microvilli, with cells becoming elongated and invasive, the formation of a new front-end back-end polarity, and the appearance of actin microfilaments and dense bodies. These morphological changes were accompanied by phenotypic changes. Double immunohistochemistry staining showed that the addition of TGF-β1 to confluent cell cultures caused a loss of the epithelial marker E-cadherin and *de novo* expression of α-SMA. An intermediate stage in transdifferentiation could be seen with hypertrophic cells expressing both E-cadherin and α-SMA. *De novo* α-SMA expression was confirmed by Northern blotting, Western blotting, and flow cytometry. In particular, cells with a transformed morphology showed strong α-SMA immunostaining of characteristic microfilament structures along the cell axis. There was a dose-dependent increase in the percentage of cells expressing α-SMA with increasing concentrations of TGF-β1, which was completely inhibited by the addition of a neutralizing anti–TGF-β1 antibody. Compared with growth on plastic, cell culture on collagen-coated plates showed a threefold increase in the percentage of cells expressing α-SMA in response to TGF-β1.

Conclusion. TGF-β1 is a key mediator that regulates, in a dose-dependent fashion, transdifferentiation of tubular epithelial cells into α-SMA+ myofibroblasts. This transdifferentiation is markedly enhanced by growth on collagen type I. These findings have identified a novel pathway that may contribute to renal fibrosis associated with overexpression of TGF-β1 within the diseased kidney.

Key words: TGF-β, transdifferentiation, collagen type 1, α-smooth muscle actin, fibrosis, tubulointerstitial fibrosis.

Received for publication August 11, 1998
and in revised form May 11, 1999
Accepted for publication May 19, 1999

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Tubulointerstitial fibrosis is considered to be the common final pathway leading to end-stage renal failure, irrespective of the nature of the initial renal injury. The process of tubulointerstitial fibrosis involves the loss of renal tubules and the accumulation of extracellular matrix (ECM) proteins, such as collagen (types I, III, IV, V, and VII), fibronectin, and laminin [1]. Although tubular epithelial cells can synthesize a variety of ECM proteins [2–4], it is thought that myofibroblasts are the main source of the increased ECM deposition seen in renal fibrosis and, indeed, in other types of tissue fibrosis [4–6]. The number of myofibroblasts identified by the expression of the α isoform of smooth muscle actin (α-SMA) is the best prognostic indicator of disease progression in both human and experimental glomerulonephritis [7–14]. Therefore, it is crucial to understand the origin of myofibroblasts and how myofibroblast production of ECM proteins is regulated.

We know little of the origin of myofibroblasts within the injured kidney. It had been suggested that interstitial myofibroblasts may derive from the differentiation of fibroblasts, the migration of perivascular smooth muscle cells or local proliferation [12, 15–17]. Another possibility, suggested by two recent studies, is that tubular epithelial cells can transdifferentiate into myofibroblasts under pathological conditions. Strutz et al showed that tubular epithelial cells can express fibroblast-specific

protein 1 (Fsp1), a fibroblast marker, in a mouse model of antitubular basement membrane disease [18]. In addition, we have found phenotypic and ultrastructural evidence of transdifferentiation of tubular epithelial cells into α-SMA+ myofibroblasts in the rat remnant kidney [19]. However, the mechanism(s) regulating the process of TEMT remains largely unknown.

Transforming growth factor-β1 (TGF-β1), a multifunctional cytokine with fibrogenic properties, has been implicated in the pathogenesis of renal fibrosis in both experimental and human glomerulonephritis [20]. This is most clearly illustrated by the deliberate overexpression of TGF-β1 within the normal kidney, which induces fibrosis [21]. TGF-β stimulates ECM deposition by increasing the synthesis of ECM proteins on the one hand while acting to inhibit their degradation on the other [20]. *In vitro*, TGF-β has been shown to induce epithelial-mesenchymal transdifferentiation of mammary epithelial cells and embryonic cardiac endothelial cells [22, 23]. Furthermore, TGF-β has been shown to stimulate collagen synthesis and induce expression of Fsp1 by cultured mouse renal proximal tubular epithelial cells [24]. These studies suggest that TGF-β may be an important inducer of epithelial-mesenchymal transdifferentiation.

In this study, we examined the ability of TGF-β1 to induce transdifferentiation of the normal rat tubular epithelial cell line (NRK52E) into cells with phenotypic and ultrastructural characteristics of myofibroblasts.

METHODS

Cell culture

The well-characterized normal rat kidney epithelial cell line (NRK52E) was obtained from the American Type Culture Collection (Rockville, MD, USA). This cell line was used in preference to primary cultures of tubular epithelial cells in order to avoid potential fibroblast contamination. NRK52E cells are thought to be of proximal tubular origin because they have a profile of collagen production consistent with proximal tubular cells, and they secrete C-type natriuretic peptide and express receptors for epidermal growth factor (EGF), but do not express EGF itself [3, 25, 26]. NRK52E cells were cultured in Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/liter glucose; Sigma Chemical Co, St. Louis, MO, USA) containing 1% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere in six-well plastic plates (Greiner Labortechnik, Frickenhausen, Germany), four-chamber glass slides, or Thermanox plastic cover slips (Nunc, Naperville, IL, USA). In some experiments, the culture plates, slides, or cover slips were coated with collagen type I (cat #150026; ICN Pharmaceuticals Inc, Costa Mesa, CA, USA). To examine transdifferentiation, cells were cultured for six days in the presence of 0, 1, 10 or 50 ng/ml recombinant human TGF-β (Promega, Madison, WI, USA) with or without 5 or 10 μg/ml of a neutralizing polyclonal rabbit anti–TGF-β1 antibody (Promega). During these experiments, the media were changed after three days, at which point fresh TGF-β1 and/or antibody were added.

Electron microscopy

For scanning electron microscopy, cells were grown on plastic cover slips, fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS; pH 7.4) for 60 minutes, rinsed with PBS, and dehydrated through graded ethanol (50, 75, 95, 100% for 10 min each). Cells then were transferred to amyl acetate for 10 minutes, put on critical point drying, and then coated with gold. Cells were viewed in a scanning electron microscope (Hitachi S-2300).

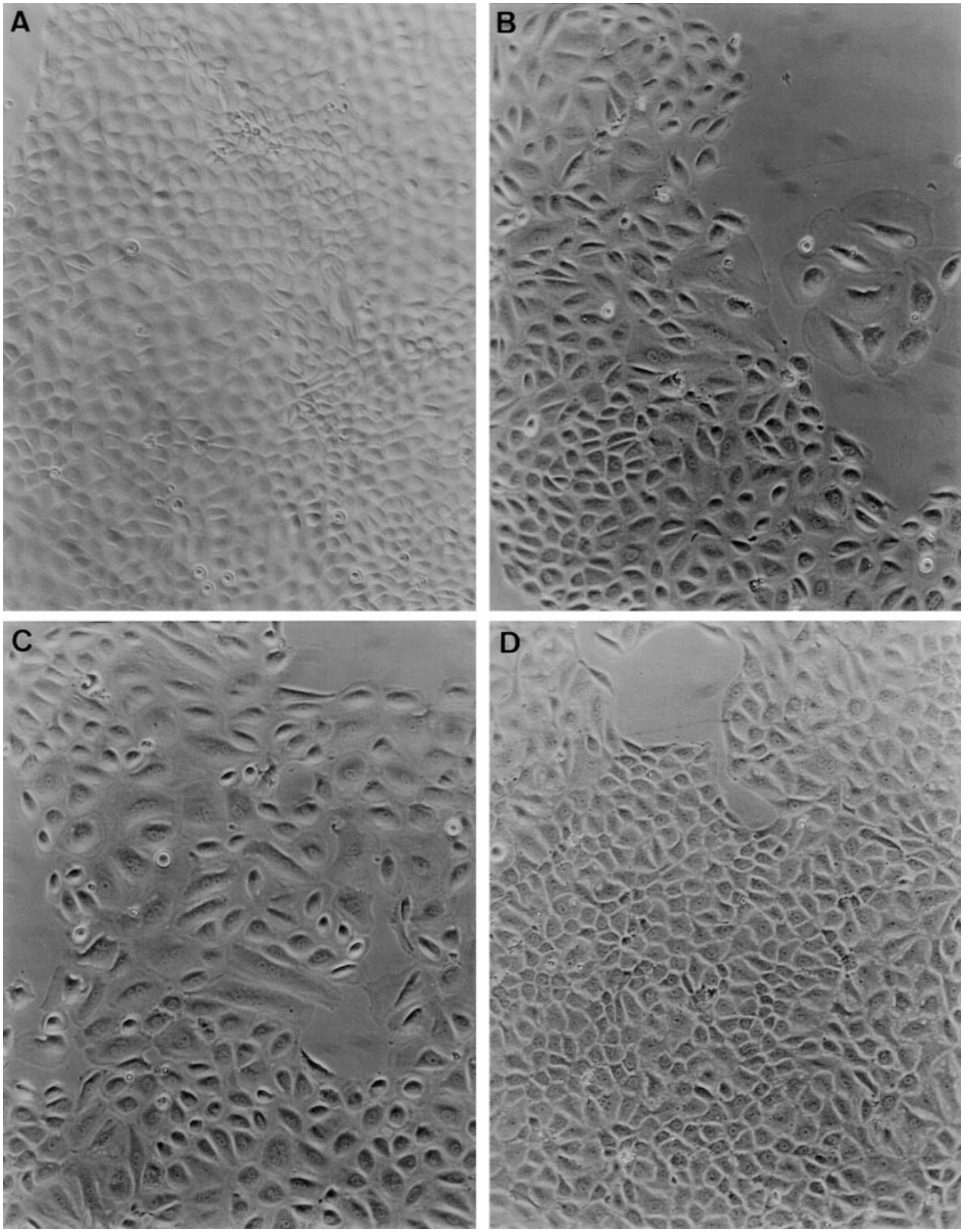
Transmission electron microscopy followed a previously described protocol [27], with some modifications. Cells were grown on cover slips fixed in 2.5% glutaraldehyde in PBS (pH 7.4), postfixed with 1% osmium tetroxide for 60 minutes, dehydrated through graded alcohols, cut into small pieces, and embedded in Epon-Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Philips CM12 electron microscope.

Northern blot analysis

A 1068 bp cDNA fragment of rat vascular smooth muscle α-actin [28] was amplified by the polymerase chain reaction and cloned using the pMOSBlue T-vector kit (Amersham International, Buckinghamshire, UK). A 358 bp cRNA riboprobe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Antisense digoxigenin (DIG)-labeled cRNA probes were prepared using a T7 RNA polymerase kit (Boehringer Mannheim GmbH, Mannheim, Germany). Probes were precipitated, and incorporation of DIG was determined by dot blotting.

NRK52E cells were grown in collagen-coated six-well plates with or without TGF-β1 or anti–TGF-β1 antibody.

Fig. 1. Phase contrast microscopy showing fibroblast-like morphological changes in cultured NRK52E tubular epithelial cells. (*A*) Cells cultured in medium alone maintained a classic cobblestone epithelial morphology and growth pattern. Cells cultured for six days with 10 ng/ml transforming growth factor-β1 (TGF-β1) (*B*), and more notably with 50 ng/ml TGF-β1 (*C*), exhibited marked hypertrophy, became elongated, and developed a highly invasive growth pattern. (*D*) The morphological changes induced by 50 ng/ml TGF-β1 were blocked by the addition of 10 μg/ml of a neutralizing anti–TGF-β1 antibody (magnification ×100).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B
C
D

Expression of α-SMA mRNA was analyzed by Northern blotting as described previously [29]. Cells were harvested by brief trypsinization and RNA extracted using RNAzol (GIBCO BRL, Gaithersburg, MD, USA). Fifteen microgram samples were denatured with glyoxal and dimethylsulfoxide, size fractionated on 1.2% agarose gels, and capillary blotted onto positively charged nylon membranes (Boehringer Mannheim). Membranes were hybridized overnight at 68°C with DIG-labeled α-SMA or GAPDH cRNA probes in DIG Easy Hyb (Boehringer Mannheim). Following hybridization, membranes were washed finally in 0.1 × standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 68°C. Bound probes were detected using sheep anti-DIG antibody (Fab) conjugated with alkaline phosphatase and development with CPD-star enhanced chemiluminescence (Boehringer Mannheim). Chemiluminescence emissions were captured on Kodak XAR film, and densitometry analysis was performed using the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD, USA). Two experiments were performed.

Western blot analysis

NRK52E cells were grown in collagen-coated six-well plates with or without TGF-β1 or anti–TGF-1 antibody. Expression of α-SMA protein in cell lysates was analyzed by Western blotting as previously described [30]. Cells were washed in PBS and then lyzed in 1 ml of 1% Nonidet P-40, 25 mM Tris-HCl, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, containing a 1 in 50 dilution of a protease inhibitor cocktail (P2714; Sigma) for 30 minutes on ice. Samples were centrifuged at 14,000 *g* for five minutes to pellet cell debris. Samples (20 μg) were mixed with a SDS-polyacrylamide gel electrophoresis sample buffer, boiled for five minutes, electrophoresed on a 10% SDS polyacrylamide gel and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham International). The membrane was blocked in PBS containing 5% skimmed milk powder, 1% FCS, and 0.02% Tween 20 and was then incubated for one hour with 1 μg/ml of 1A4 anti–α-SMA mAb (Sigma) diluted in the previously mentioned buffer. After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat antimouse IgG in PBS containing 1% normal goat serum and 1% FCS. The blot was then developed using the ECL detection kit (Amersham) to produce a chemiluminescence signal that was captured on x-ray film. Three experiments were performed.

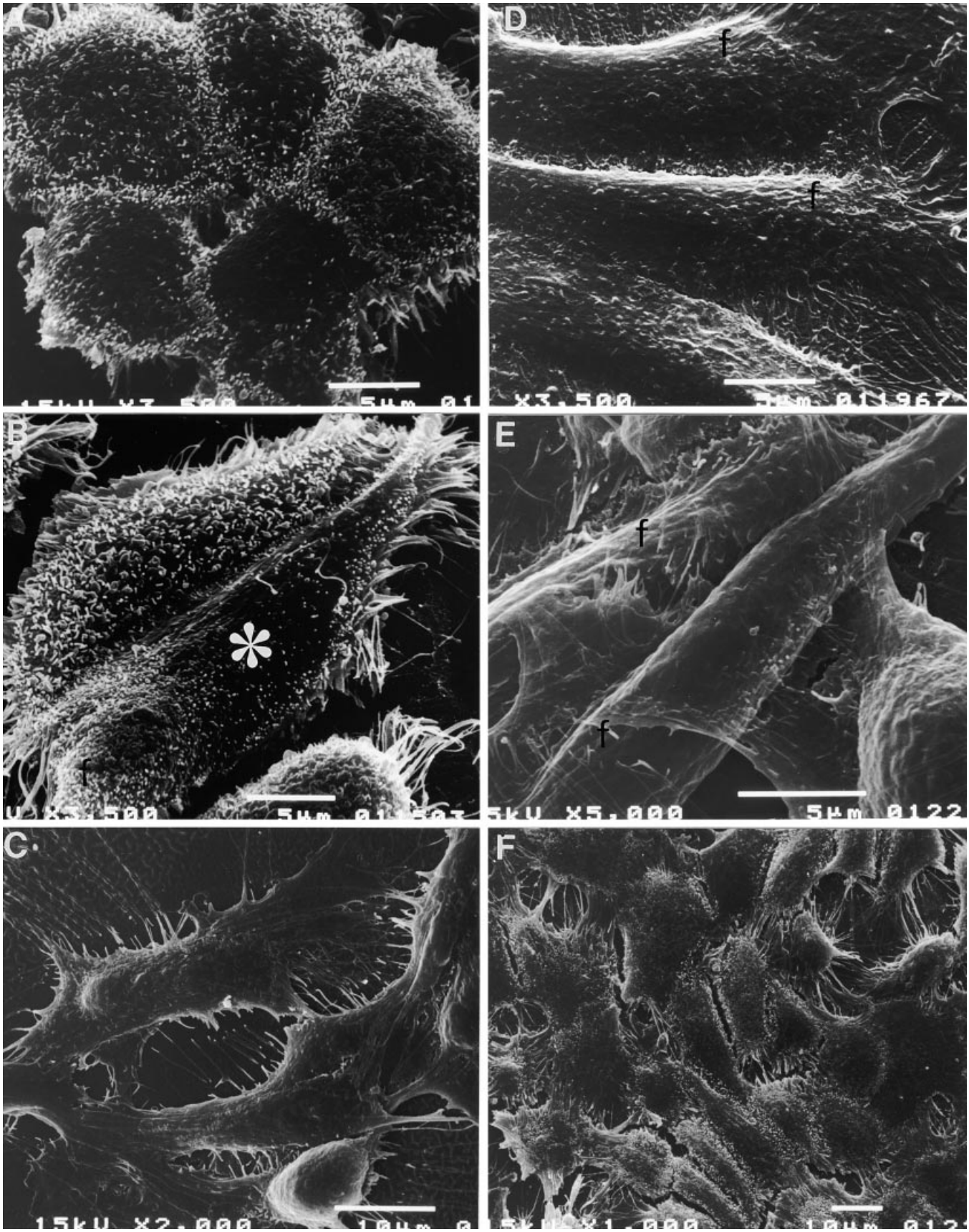
Flow cytometry

A technique developed for the detection of intracellular antigens [31] was used to detect α-SMA within transformed NRK52E cells. NRK52E cells were grown in the presence or absence of TGF-β, harvested by brief trypsinization, washed in cold PBS, fixed in 2% paraformaldehyde in PBS for 30 minutes on ice followed by permeabilization with 0.2% saponin (Sigma) for 10 minutes. After washing, cells were stained with the 1A4 anti–α-SMA mAb (1:2000) or an isotype control mAb 73.5 (antihuman CD45R antigen) for 20 minutes in 0.2% saponin and washed and then labeled with FITC-conjugated goat antimouse IgG for 15 minutes in 0.2% saponin. All samples were analyzed on an MoFlo cytometer connected to a CICERO data acquisition system (Cytomation, Fort Collins, CO, USA). Fluorescence histograms of at least 5000 counts were generated from a gate set in the forward angle versus 90° light scatter scattergram. The percentage of positive cells was measured from a cut-off set using the isotype-matched nonspecific control antibody, whereas the mean channel fluorescence (MCF) was measured over the entire distribution. Data are expressed as the percentage of $\alpha\text{-SMA}^+$ cells or the intensity of the MCF ± SEM from five experiments.

Immunohistochemistry

NRK52E cells were cultured in four-chamber glass slides with or without collagen coating in the presence or absence of TGF-β, and were stained with monoclonal antibodies (mAbs) to α-SMA or E-cadherin (C37020; Transduction Laboratories, Lexington, KY, USA) using one- or two-color immunohistochemistry, as previously described [19]. Cells were rinsed in PBS, fixed in 2% paraformaldehyde, and preincubated with 10% FCS and 10% normal sheep serum to block nonspecific binding and were then incubated with the 1A4 anti–α-SMA or irrelevant isotype control mAb (73.5) for 60 minutes. After washing with PBS, endogenous peroxidase was inactivated by incubation in 0.3% H_2O_2 in methanol for

Fig. 2. Scanning electron microscopy showing tubular epithelial-myofibroblast transdifferentiation (TEMT). NRK52E cells were cultured for six days on collagen-coated cover slips in the presence or absence of TGF-β1. (*A*) Cells grown in medium alone show a normal aggregated epithelial growth pattern with apical-basal polarity and many microvilli on the cell surface. (*B*) Culture in 1 ng/ml TGF-β1 showed partial, but incomplete, transdifferentiation with the development of mild hypertrophy associated with an elongated morphology and a partial loss of microvilli on the cell surface (*). (*C–E*) Culture in 50 ng/ml TGF-β1 induces complete tubular epithelial-myofibroblast transdifferentiation. Cells with a loss of apical-basal polarity, loss of microvilli, development of a highly elongated morphology, and the presence of a disaggregated and invasive growth pattern is shown in (C). The new front-end, back-end morphology with many cytoplasmic projections spreading from the new front end of cells is shown in (D). A typical example of a transformed cell with marked hypertrophy, an elongated shape, and exhibiting a new front-end, back-end morphology with complete loss of cell-surface microvilli is shown in (E). (*F*) The addition of 10 μg/ml of a neutralizing anti–TGF-β1 antibody abrogates the morphological changes induced by 50 ng/ml TGF-β1. F, front-end.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20 minutes, incubated sequentially with peroxidase-conjugated goat antimouse IgG and peroxidase-conjugated anti-peroxidase complexes (PAP), followed by development with diaminobenzidine to produce a brown color. To block antibody cross-reactivity and facilitate antigen retrieval, sections were treated with 10 minutes of microwave oven heating in 10 mM sodium citrate, pH 6.0, at 2450 MHz and 800 W power. After microwave treatment and preincubation as described earlier here, sections were then incubated with the anti–E-cadherin or control mAb for 60 minutes, followed by alkaline phosphatase-conjugated goat antimouse IgG and alkaline phosphatase-conjugated anti-alkaline phosphatase complexes (APAAP). Finally, sections were developed with Fast Blue BB Base (Sigma Chemical Co.). Cells were counterstained with hematoxylin and cover slipped in an aqueous mounting medium. All procedures were performed at room temperature.

For quantitative analysis of α-SMA and E-cadherin expression, NRK52E cells were cultured for six days in six-well plastic plates with or without collagen coating. Cells were then harvested by brief trypsinization and fixed in 2% paraformaldehyde, and cell spots were prepared on silanated slides. Immunoperoxidase staining with the mAbs was performed as described earlier in this study.

Quantitation of immunohistochemical staining

The number of NRK52E cells stained with the anti–α-SMA or anti–E-cadherin mAbs was determined by counting the number of positive-stained cells in a total of at least 1000 cells under high power (×400) in each cell spot. Data from five experiments are expressed as the mean percentage ± SEM. For all analyses, the observer was blinded to the coded slides.

Statistical analysis

Data obtained from this study were analyzed by one-way analysis of variance using the Newman–Keuls Multiple Comparison Test from the Complete Statistical Analysis program (CSS; Statsoft, Tulsa, IL, USA).

RESULTS

Morphological demonstration of transforming growth factor-β1–induced tubular epithelial-myofibroblast transdifferentiation by NRK52E cells

NRK52E cells cultured in six-well plates or four-chamber glass slides produced a confluent monolayer with a cobblestone morphology. Growth of NRK52E cells on collagen-coated plates, or slides, also produced a confluent monolayer of cells with a classical epithelial cobblestone morphology (Fig. 1A). Scanning and transmission electron microscopy demonstrated that these cells had a cubic or round-shape, displayed apical-basal polarity, and had many microvilli on the cell surface (Figs. 2A and 3A). There was no evidence of actin-like microfilaments within these cells (Fig. 3A).

The culture of NRK52E cells in the presence of TGF-β1 for six days induced a clear fibroblast-like morphology. These changes were particularly marked when cells were grown on collagen-coated plates or slides and were dose dependent (Figs. 1 B, C and 2 B, C). Culture in 1 ng/ml TGF-β1 caused no change in NRK52E cell morphology by phase contrast microscopy (data not shown). However, scanning electron microscopy showed the presence of occasional cells exhibiting mild hypertrophy and starting to develop a spindle-like shape, but which retained epithelial polarity and microvilli (Fig. 2B). Culture of NRK52E cells in 10 ng/ml TGF-β1 induced profound morphological changes, with cells developing marked hypertrophy, becoming elongated, and losing the cobblestone growth pattern (Fig. 1B). The number of cells undergoing morphological transformation was further increased when TGF-β1 was added at a dose of 50 ng/ml (Fig. 1C). Scanning electron microscopy showed that the transformed cells completely lost epithelial polarity and microvilli on the cell surface. They became elongated, disassociated with neighboring cells, and were highly invasive with many cytoplasmic projections (Fig. 2C). The transformed cells also developed a new front-end back-end fibroblast-like polarity, with many cytoplasmic projections present at the new front end (Fig. 2 C–E). Transmission electron microscopy demonstrated that TGF-β1 induced differentiation of NRK52E cells into cells of a typical myofibroblast morphology, with the presence of large bundles of actin microfilaments and dense bodies within the cytoplasm (Fig. 3C). These actin microfilaments and dense bodies were seen along the cell axis and within the cytoplasmic projections at the new front end of the transformed cells (Fig. 3 C, D).

The ability of TGF-β1 to induce the transformation of NRK52E cells was inhibited by the addition of a neutralizing anti–TGF-β1 antibody, with cells retaining epithelial polarity, microvilli, tight junctions, a cobblestone growth pattern, with no evidence of hypertrophy, an elongated morphology, or actin microfilaments (Figs. 1F, 2F, and 3B).

Phenotypic demonstration of transforming growth factor-β1 induced tubular epithelial-myofibroblast transdifferentiation by NRK52E cells

Northern blot analysis showed a single, faint band for α-SMA mRNA in cultures of NRK52E cell grown in medium with 1% FCS on collagen-coated plates (Fig. 4). The addition of TGF-β1 induced a dose-dependent increase in α-SMA mRNA expression, with a maximal sevenfold increase over unstimulated cells. This was a specific response, as demonstrated by blockade with a neutralizing anti–TGF-β1 antibody (Fig. 4). Western

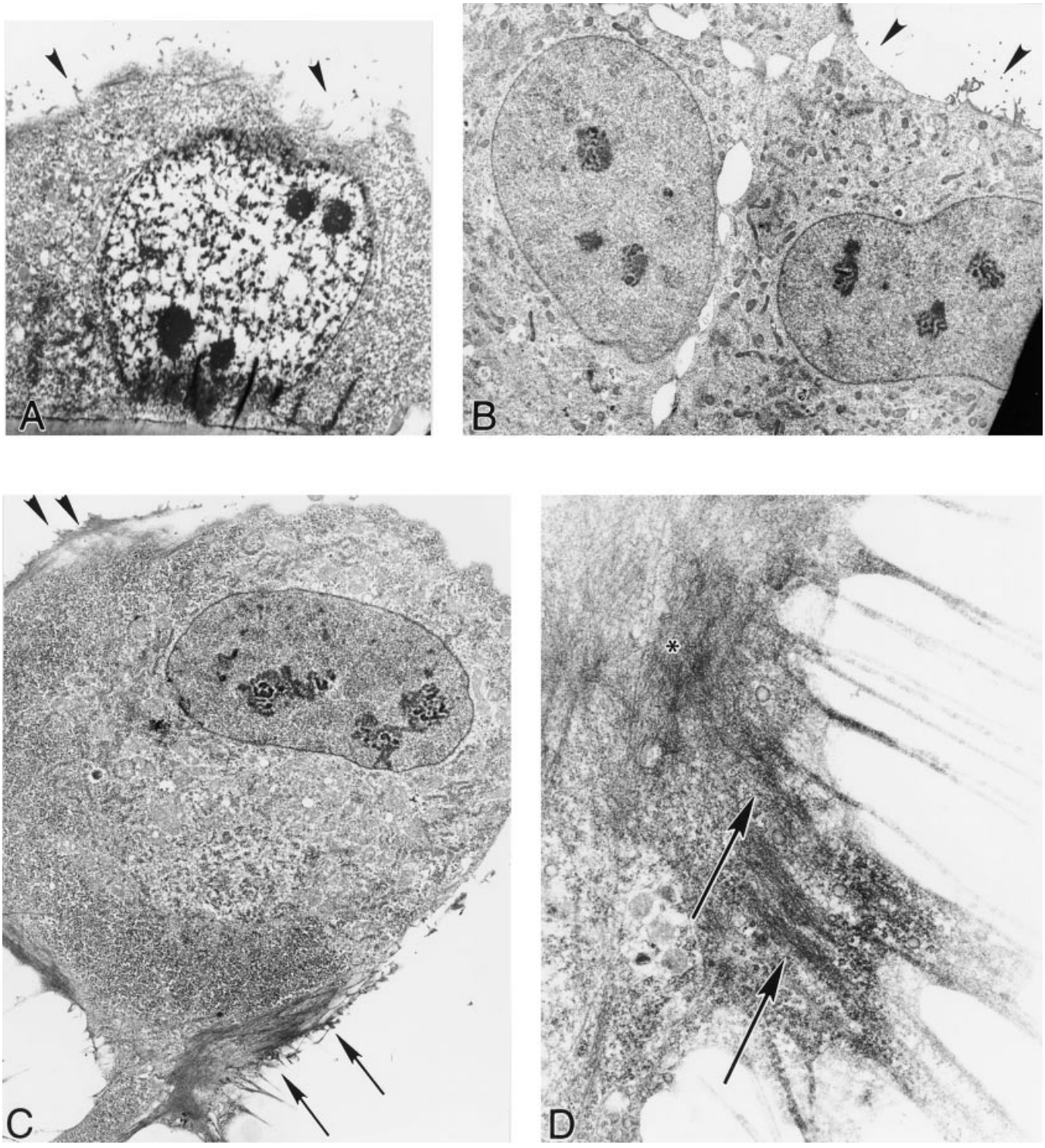


Fig. 3. Transmission electron microscopy showing tubular epithelial-myofibroblast transdifferentiation (TEMT). NRK52E cells were cultured for six days on collagen-coated cover slips in the presence or absence of TGF-β1. (*A*) Cells grown in medium alone show a normal epithelial morphology with apical-basal polarity and many microvilli (arrowheads) on cell surface. (*B*) The addition of 10 μg/ml of a neutralizing antibody anti–TGF-β1 antibody almost completely blocked the morphological changes induced by 50 ng/ml of TGF-β1 (*C* and *D*). In particular, the NRK52E cells maintained apical-basal polarity, microvilli on the cell surface (arrowheads), and tight junctions. (C and D) Cells grown in the presence of 50 ng/ml TGF-β1. (C) A cell in the early stage of transformation shows hypertrophy, an elongated morphology, and a loss of apical-basal polarity, microvilli, and tight junctions. There are prominent bundles of actin microfilaments lying peripheral to the cytoplasm at the new front-end (arrowheads) and back-end of the cell. (D) A cell at a later stage of transformation showing characteristic actin microfilament bundles (arrows) with dense bodies (*) throughout the cytoplasm, forming microfilament-rich cytoplasmic projections at the front end of the cell. Magnifications (A) ×4800, (B and C) ×4200, and (D) ×18,300.

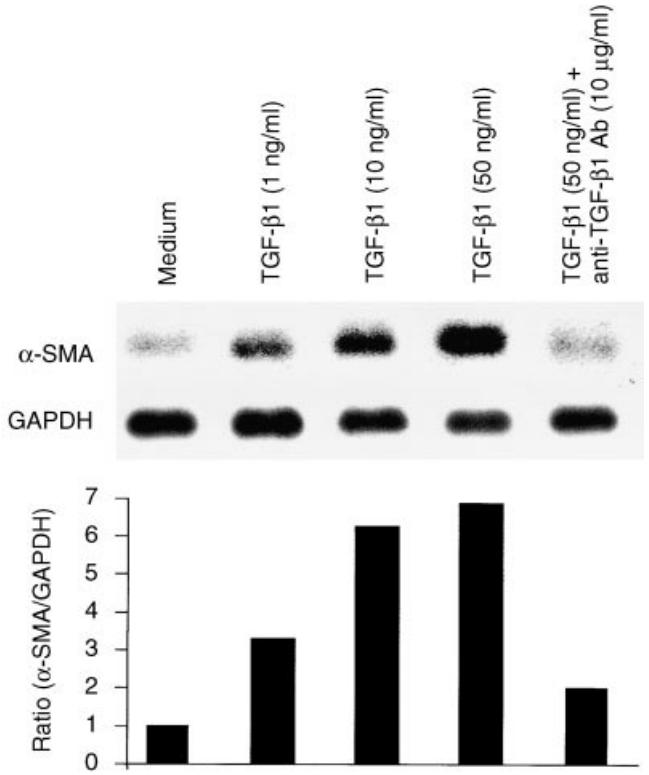


Fig. 4. Northern blot analysis of α-smooth muscle actin (α-SMA) mRNA expression by NRK52E cells. Cells were cultured for six days in collagen-coated six-well plates in the presence of medium alone, TGF-β1, or TGF-β1 plus a neutralizing anti–TGF-β1 antibody. The blot was probed for α-SMA and GAPDH expression. The ratio of α-SMA/GAPDH ratio is shown in the graph. This is one of two experiments that produced similar results.

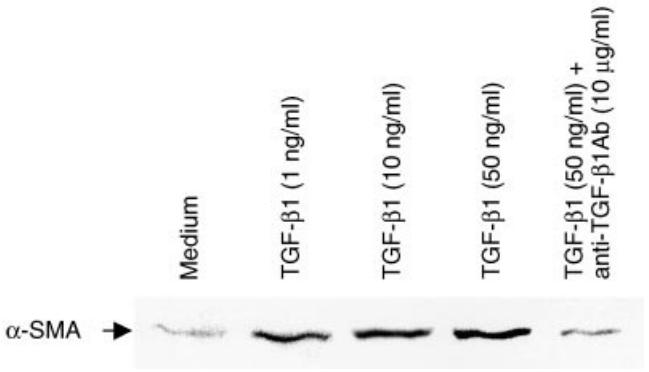


Fig. 5. Western blot analysis of α-smooth muscle actin (α-SMA) protein expression by NRK52E cells. Cells were cultured for six days in collagen-coated six-well plates in the presence of medium alone, TGF-β1, or TGF-β1 plus a neutralizing anti–TGF-β1 antibody. A single band of approximately 43 kDa was detected. This is one of three experiments that gave similar results.

blotting of cell lysates showed that TGF-β1 induced a dose-dependent increase in the expression of α-SMA protein in NRK52E cells, which was also inhibited by the addition of a neutralizing antibody (Fig. 5).

Flow cytometry of saponin-permeabilized NRK52E

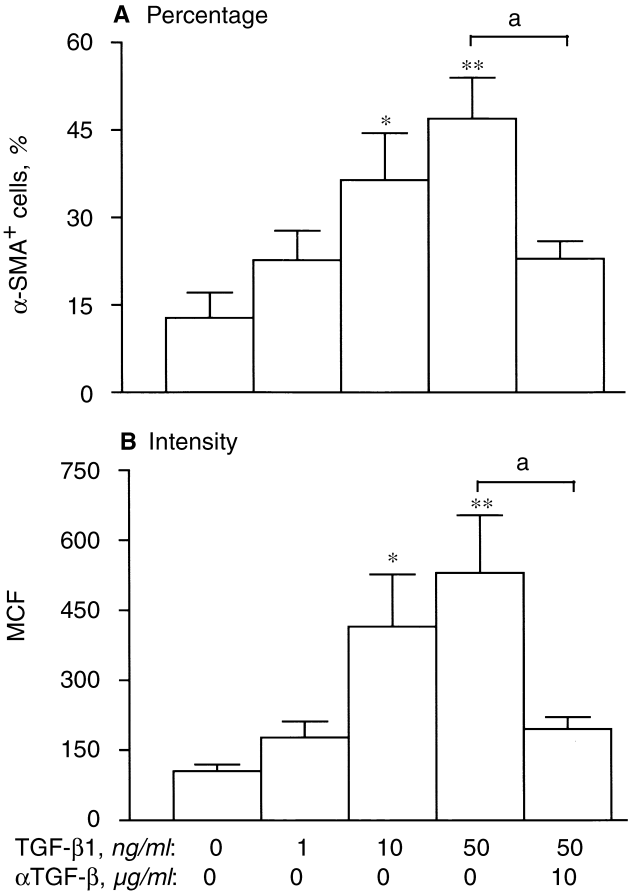
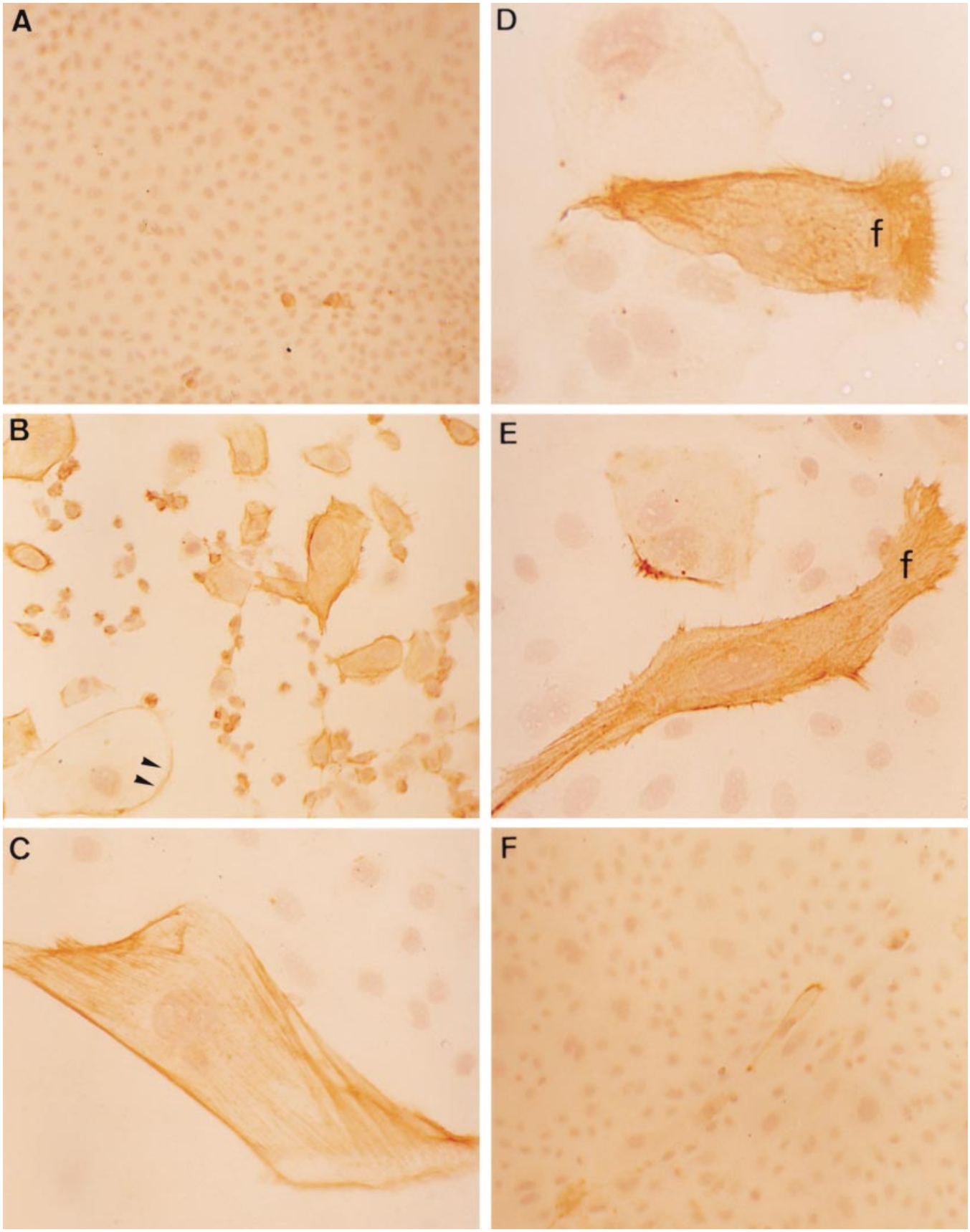


Fig. 6. Flow cytometric analysis of α-SMA expression by NRK52E cells. Cells were cultured for six days in collagen-coated six-well plates in the presence of medium alone, TGF-β1, or TGF-β1 plus a neutralizing anti–TGF-β1 antibody. (*A*) The percentage and (*B*) the mean channel fluorescence intensity (MCF) of α-SMA expression are shown. The background fluorescence signal from the irrelevant control antibody has been subtracted. Each bar represents the mean ± SEM from five separate experiments. $^{*}P < 0.05$; $^{**}P < 0.001$ compared with cells grown in medium alone; $^{a}P < 0.05$.

Fig. 7. Combined phenotypic and morphologic demonstration of tubular epithelial-myofibroblast transdifferentiation (TEMT). NRK52E cells were grown for six days on collagen-coated glass slides in the presence or absence of TGF-β1. Cells expressing α-SMA protein were detected by immunoperoxidase staining (brown) using the 1A4 mAb. (*A*) Cells cultured in medium alone show a normal epithelial morphology with a cobblestone grown pattern. Only occasional cells stained positive for α-SMA. (*B*) Culture in 50 ng/ml TGF-β1 induced TEMT. Most of the cells expressing α-SMA (brown stain) exhibited hypertrophy, an elongated morphology, a disaggregated and invasive growth pattern, and a new front-end back-end mesenchymal polarity. Further examples of transformation are: (*C*) a cell in the early stage of transformation showing marked hypertrophy and numerous α-SMA+ myofilaments within the cytoplasm along the cell axis; (*D*) a transformed cell showing a new front-end back-end polarity, with many α-SMA+ cytoplasmic projections at the new front-end; and (*E*) a highly elongated, hypertrophic, and invasive myofibroblast showing strong α-SMA expression. (*F*) The addition of 10 μg/ml of a neutralizing anti–TGF-β1 antibody completely blocked TEMT induced by 50 ng/ml TGF-β1. Magnifications ×100 (A, B, and F), ×400 (C, D, and E).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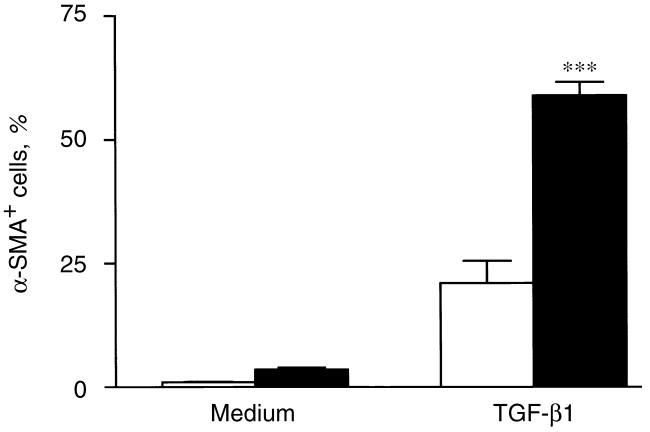

Fig. 8. Effect of collagen type I on TGF-β1 induced α-smooth muscle actin expression by NRK52E cells. Immunohistochemistry staining for α-SMA expression was performed on NRK52E cells cultured for six days in plastic wells (□) or collagen-coated wells (■) with or without 50 ng/ml TGF-β1. Each bar represents the mean percentage of α-SMA–positive cells ± SEM from four separate experiments. ***$P <$ 0.001 compared with growth on plastic.

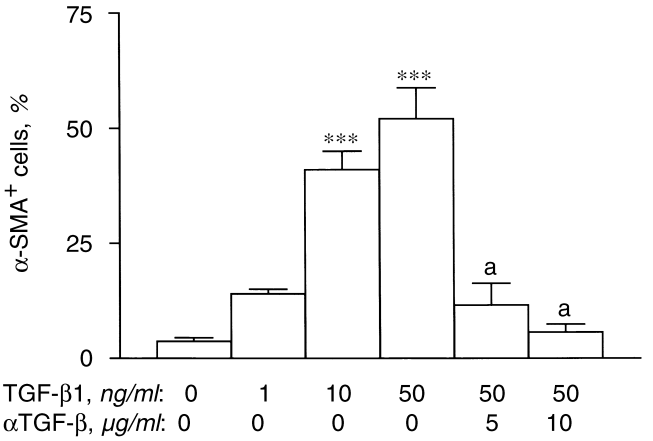

Fig. 9. TGF-β1 induces α-smooth muscle actin expression by NRK52E cells in a dose-dependent fashion. Immunohistochemistry staining for α-SMA expression was performed on NRK52E cells cultured for six days on collagen-coated wells in the presence of varying concentrations of TGF-β1 with or without a neutralizing anti–TGF-β1 antibody. Each bar represents the mean percentage of α-SMA–positive cells ± SEM from four separate experiments. ***$P < 0.001$ compared with medium alone; [a]$P < 0.001$ compared with 50 ng/ml TGF-β1.

cells was used to quantitate TGF-β1–induced α-SMA protein expression. As shown in Figure 6 NRK52E cells grown in medium alone on collagen-coated plates exhibited a very weak expression of α-SMA protein. The addition of TGF-β1 caused a dose-dependent increase in the percentage of NRK52E cells expressing α-SMA, with almost half (46.8 ± 7%) of the cells being positive when cultured in 50 ng/ml TGF-β1 (Fig. 6A). As well as increasing the percentage of NRK52E cells expressing α-SMA protein, TGF-β1 caused a dose-dependent increase in the intensity of α-SMA expression (Fig. 6B). The ability of 50 ng/ml TGF-β1 to increase α-SMA protein expression was almost completely abrogated by the addition of a neutralizing antibody (Fig. 6).

Immunohistochemistry staining was used to evaluate the morphology and number of NRK52E cells expressing α-SMA protein. NRK52E cells cultured on plastic or collagen-coated surfaces showed a normal epithelial morphology and cobblestone growth pattern, with only occasional cells staining positive for α-SMA (Fig. 7A). Those NRK52E cells induced to transdifferentiate by TGF-β1, whether grown on plastic or collagen-coated surfaces, all exhibited strong α-SMA staining (Fig. 7 B–F). The various elements of TGF-β1–induced transdifferentiation could be seen in α-SMA–stained cells. For example, α-SMA–positive cells exhibited hypertrophy, an elongated morphology, and an invasive growth pattern (Fig. 7B). Transformed cells also showed strong α-SMA staining in cytoplasmic myofilaments along the cell axis and in the many cytoplasmic projects seen at the new front end of cells with an invasive mesenchymal polarity (Fig. 7 C–E).

The number of NRK52E cells staining positive for α-SMA was also quantitated (Figs. 8 and 9). An interesting observation was that NRK52E cells grown on collagen-coated plates showed a threefold increase in the percentage of cells expressing α-SMA cells following TGF-β1 stimulation compared with cells grown on plastic plates (Fig. 8). TGF-β1 induced a dose-dependent increase in the percentage of NRK52E cells expressing α-SMA, which was completely abrogated by the addition of a neutralizing anti–TGF-β1 antibody (Figs. 7f and 9).

As a second phenotypic marker of TGF-β1–induced transdifferentiation of NRK52E cells, we analyzed the expression of the epithelial antigen E-cadherin. Confluent cultures of NRK52E cells showed E-cadherin expression along the cell surface at tight junctions (Fig. 10a). Culture of cells in 10 ng/ml TGF-β1 caused *de novo* expression of α-SMA in hypertrophic cells, many of which maintained E-cadherin expression (Fig. 10b). Culture of cells in higher concentrations of TGF-β1 caused a more profound change in morphology, and these cells showed strong α-SMA expression with a complete loss of E-cadherin expression (Fig. 10c). Quantitation of the percentage of α-SMA and E-cadherin positive cells after six days of culture in 50 ng/ml TGF-β1 showed a largely reciprocal pattern of expression of these two markers (Fig. 11). These phenotypic changes were prevented by addition of a neutralizing anti–TGF-β1 antibody (Fig. 10d and 11).

DISCUSSION

This study provides evidence that TGF-β1 can induce complete transdifferentiation of the normal rat tubular epithelial cell line (NRK52E) into myofibroblasts on the

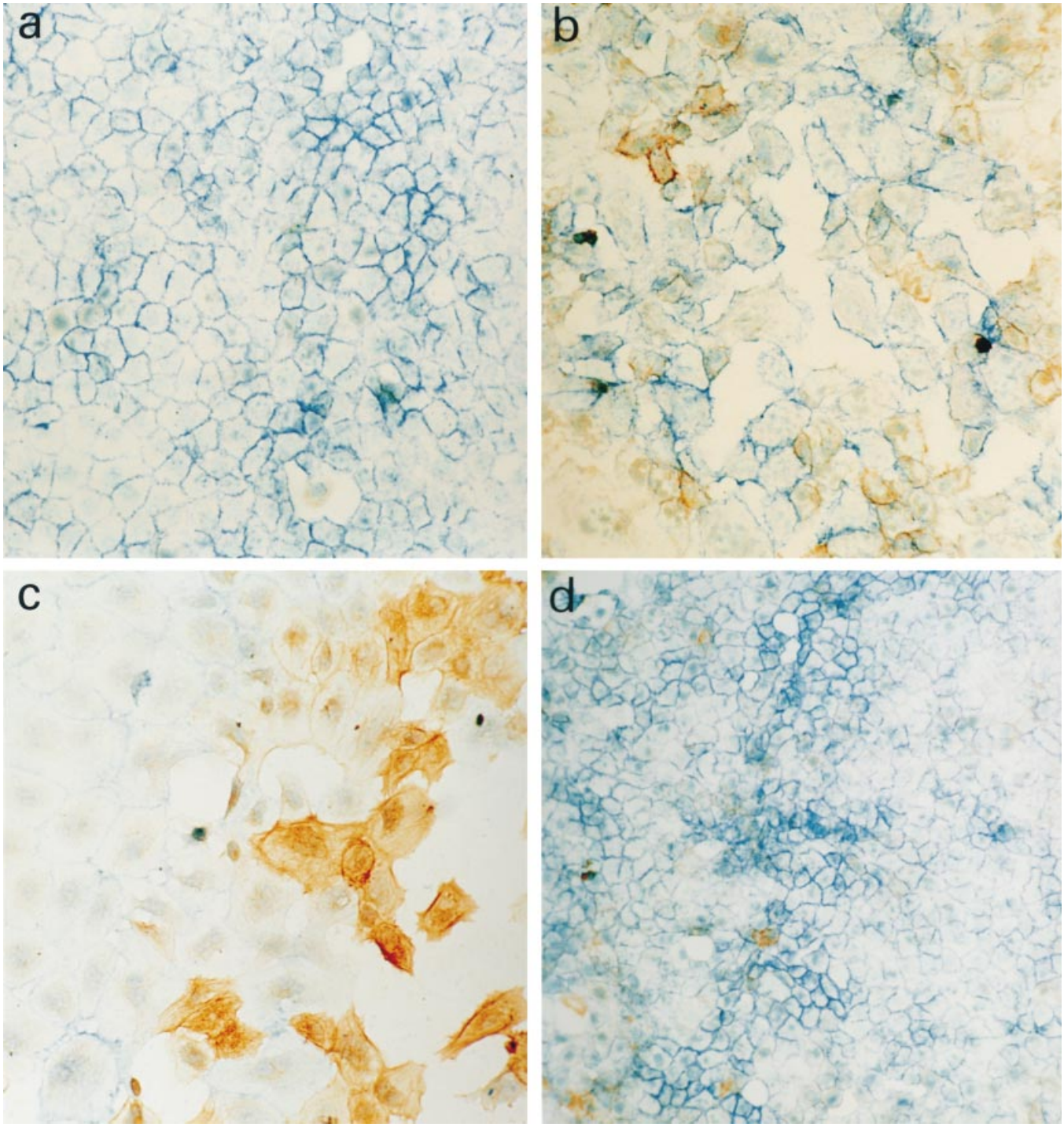

Fig. 10. TGF-β1 induces loss of E-cadherin expression and *de novo* expression of α-SMA in NRK52E cells. Double immunostaining was used to detect E-cadherin (blue) and α-SMA (brown). (*a*) Confluent, cobblestone cells in control medium showing E-cadherin (blue) expression in tight junctions and no α-SMA expression. (*b*) Cells cultured for six days in 10 ng/ml TGF-β1 causes the induction of α-SMA and a loss of E-cadherin expression. Indeed, many double-positive cells can be seen which also exhibit hypertrophy with an irregular growth pattern. (*c*) Cell culture for six days in 50 ng/ml TGF-β1 causes a marked induction of α-SMA (brown) and a loss of E-cadherin expression by cells that have lost the epithelial morphology, becoming large and elongated. Note that weak E-cadherin expression is maintained in untransformed cells that retain a cobblestone pattern. (*d*) The addition of a neutralizing anti–TGF-β antibody inhibits the changes seen with 50 ng/ml TGF-β. Only occasional α-SMA–positive cells are seen, whereas virtually all cells retain E-cadherin expression at tight junctions in cells with a cobblestone growth pattern. Magnification ×200 (a–c), ×160 (d).

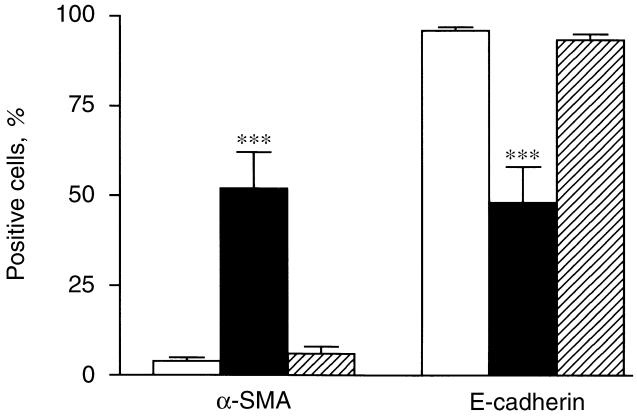

Fig. 11. Quantitation of E-cadherin and α-SMA expression following TGF-β1 addition to NRK52E cells. Double immunohistochemistry staining for E-cadherin and α-SMA expression was performed on NRK52E cells cultured for six days on collagen-coated wells in the presence of varying concentrations of TGF-β1 with or without a neutralizing anti–TGF-β1 antibody. Symbols are: (□) medium; (■) TGF-β1; (▨) TGF-β1 + antibody. Each bar represents the mean percentage of positive cells ± SEM from four separate experiments. ***$P < 0.001$ compared with medium alone.

basis of ultrastructural morphology, *de novo* expression of the mesenchymal marker, α-SMA, and the loss of expression of the epithelial marker E-cadherin. This process is dose dependent and is substantially augmented by cell growth on collagen type I. These *in vitro* studies lend support to recent reports describing the transdifferentiation of tubular epithelial cells into fibroblasts, or myofibroblasts, in experimental kidney disease [18, 19].

An important observation in this study was that TGF-β1–induced transdifferentiation of NRK52E cells was dose dependent. This was not just a simple matter of adding more TGF-β1 to get a larger number of cells to transdifferentiate, but there was a threshold concentration of TGF-β1 that had to be reached in order to achieve complete transdifferentiation. This was illustrated by a culture of cells in 1 ng/ml TGF-β1 that induced a minor increase in α-SMA mRNA and protein expression but failed to drive a complete morphological transformation. Indeed, phase-contrast microscopy of these cells showed little morphological change. However, electron microscopy revealed that some cells had become elongated, showed a partial loss of surface microvilli, and had developed a limited organization of stress fibers, but they did not achieve complete transdifferentiation in terms of developing a new front-end back-end polarity or an invasive growth pattern. The development of complete morphological transformation of NRK52E cells required culture in 10 ng/ml TGF-β1. Increasing the concentration of TGF-β1 from 10 to 50 ng/ml increased the number of cells undergoing transdifferentiation, without changing the morphology of the transformed cells.

The threshold concentration of TGF-β1 required to drive TEMT may provide an explanation for some of the apparently contradictory results in the literature. For example, a study by Creely et al found that NRK52E cells cultured for five days in 3 ng/ml TGF-β1 caused no change in the overall cobblestone pattern of cell growth [3]. However, primary cultures of rabbit proximal tubular epithelial cells grown in 10 ng/ml TGF-β1 for 48 hours did show marked transformation, with cells becoming elongated, developing actin stress fibers, and forming clusters of adherent cells [32, 33]. Interestingly, the use of 3 ng/ml TGF-β1 caused partial, but not complete, morphological transformation of the mouse proximal tubular epithelial cell line [24]. Indeed, a combination of EGF plus TGF-β1 was required to cause complete epithelial-mesenchymal transdifferentiation, characterized by spindle-shaped cells with stress fibers, a loss of epithelial-specific antigens, and the expression of mesenchymal antigens Fsp1 and α-SMA [24]. It would be interesting to know whether this additive effect is still evident when using higher concentrations of TGF-β1.

A second important finding in this study was that culture of NRK52E cells on collagen type I-coated surfaces enhanced TGF-β1–induced transdifferentiation, although collagen coating in itself did not trigger transdifferentiation. This is consistent with previous studies in which epithelial cell contacts with ECM proteins, such as collagen type I, play an important role in the induction of the transdifferentiation process [34]. For example, anterior lens epithelium with an intact basement membrane grown on top of collagen-coated plates retains a normal epithelial morphology, but when these explants are grown within a three-dimensional collagen gel, the epithelial cells transform into fibroblast-like mesenchymal cells [35]. Similarly, the culture of MCT proximal tubular epithelial cells on collagen I-coated plates induces expression of Fsp1, but not morphological transformation [24], but when MCT cells or Madin-Darby canine tubular epithelial cells are grown within three-dimensional collagen type I gels, they undergo transdifferentiation into fibroblast-like mesenchymal cells [19, 36]. Consistent with these *in vitro* studies, we found that that the transdifferentiation of tubular epithelial cells into α-SMA+ myofibroblasts in the rat remnant kidney was invariably associated with disruption of the tubular basement membrane [19], suggesting that contacts between the tubular epithelial cell and interstitial ECM components promote TEMT *in vivo.*

In summary, this study demonstrated that TGF-β1 can induce, in a dose-dependent fashion, the transdifferentiation of normal tubular epithelial cells into myofibroblasts *in vitro.* The contact between epithelial cells and collagen type I markedly enhanced TGF-β1–driven TEMT, and this may be an important element of TEMT *in vivo.* These findings have identified a novel pathway that may contribute to renal fibrosis associated with overexpression of TGF-β1 within the diseased kidney.

ACKNOWLEDGMENTS

This study was supported by the Chinese National Natural Scientific Foundation (39600071), the Taiwan National Science Council (87-2314-B075-048), and the Australian Kidney Foundation (G13N/18). The authors would like to thank Mr. Paul Hutchinson for assistance with the flow cytometry studies, Mr. Paul Crammer for assistance with the ultrastructural studies, and Ms. Rita Foti for preparation of the α-SMA probe. Parts of this work was presented at the 34th Annual Meeting of the Australian and New Zealand Society of Nephrology, Aukland, Australia, 1998.

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