Regulation of osteopontin expression in rat mesangial cells

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Abstract Hypercellularity and accumulation of extracellular matrix are common responses of renal glomeruli to inflammatory stimuli. Using the differential display approach, we compared the gene expression patterns of proliferating and differentiating rat mesangial cells in two- and three-dimensional cultures. Osteopontin, an extracellular matrix protein, was found to be transcribed, synthesized, and secreted by rat mesangial cells. Osteopontin transcription was not associated with cell proliferation and was found to be FCS-inducible in proliferating cells. Osteopontin expression was independent of exogenously supplied FCS in differentiating cells. The presented data indicate that osteopontin is differentially regulated in proliferating and differentiating mesangial cells.

Key words: Differential display; Osteopontin; Proliferation; Differentiation; Rat mesangial cell

1. Introduction

Renal mesangial hypercellularity plays an important pathogenic role in glomerular injury. Previous studies have demonstrated specific effects of soluble factors and extracellular matrix components on the mesangial cell phenotype in vivo and in vitro [1–3].

We hypothesize that signals arising from the interaction between mesangial cells, matrix molecules, and soluble factors finally lead to the induction or suppression of genes which control mesangial cell proliferation. We intended to identify genes which were regulated when the mesangial cell phenotype changed from a mitotically quiescent to a proliferative one. As experimental model, we used the three- (3D-) and two-dimensional (2D-) culture systems. As previously shown, cultured rat mesangial cells embedded within a 3D-collagen I gel exhibited a quiescent and differentiated cell phenotype, closely resembling the phenotype observed in vivo in healthy glomeruli [4,5]. In 2D-culture mesangial cells proliferated, thus resembling a phenotype observed in vivo in glomerular diseases [2,6,7].

Employing the differential display strategy [8], we compared the gene expression patterns of these two different mesangial cell phenotypes at various time points. We identified osteopontin, an extracellular matrix protein, that was dramatically downregulated in proliferating cells. In this study we investigated osteopontin expression at the mRNA and protein level in more detail.

2. Materials and methods

2.1. Cell culture

Rat mesangial cells were obtained and cultured as previously described [4,9]. Cells between the 10th and 19th passage were used for the experiments.

Collagen type I substrate for 2D- and 3D-cultures: Acid-soluble collagen type I was isolated from fetal calf skin and purified as previously described [10].

For experiments in 2D-culture dishes were coated overnight at 4°C with 12.5 μg/ml type I collagen dissolved in PBS. 16 000 cells/cm^2 were seeded per dish, cultured in 10% FCS-containing DMEM and harvested for RNA extraction at the time points indicated.

For experiments in 3D-culture, type I collagen was solubilized at 4°C in 100 mM acetic acid to achieve a final concentration of 1.25 mg/ml. Solubilized collagen I was mixed with one tenth of 10 × Earle's salt (Life Technologies, Eggenstein, Germany) and neutralized with sterile sodium hydroxide (1 M). Cells were added to achieve a final concentration of 10^6 cells/ml neutralized collagen. Polymerized collagen gels were supplied with 10% FCS-containing DMEM.

2.2. RNA isolation and Northern blotting

Total RNA was isolated according to Chomczynski and Sacchi [11]. For RNA extraction of cells in 3D-culture, the cell-containing gels were shock-frozen in liquid nitrogen, ground and dissolved in 4 M guanidinium thiocyanate. 10 μg total RNA was loaded per lane onto formaldehyde-containing agarose gels (1.2%). Northern blotting was performed as described [12]. All experiments were done at least twice.

2.3. Differential display and sequencing

Differential display was performed according to the protocol published by Liang and Pardee [8] as described before [12]. PCRs were performed using 5 μM 3′-primer ($dT_{10}MA$) and 2 μM 5′-primer (5′-TCGATACATG-3′) as primer pair [12]. Fragments of interest were isolated, reamplified, and subcloned into the *Srf*I site of pCR-Script cloning vector (Stratagene, Heidelberg, Germany).

Sequence analysis was performed according to Sanger et al. [13] using a T7-polymerase kit (Pharmacia, Freiburg, Germany).

2.4. Immunocytochemistry

For double staining of the Thy1.1 antigen and the osteopontin protein in 2D-culture, 10 000 cells were seeded into chamber slides and grown in 10% FCS-containing DMEM for 24 h. After washing, the cells were incubated in 50 mM ammonium chloride, permeabilized in 0.2% (v/v) Triton X-100, and blocked with FCS. The primary antibodies anti-osteopontin (rabbit anti-rat, diluted 1:500) and the anti-Thy1.1 antibody (mouse anti-rat, 1:1000) were added simultaneously, detected by incubation with DTAF-conjugated goat anti-rabbit and Cy3-conjugated sheep anti-mouse secondary antibodies, and visualized by fluorescence microscopy. For osteopontin staining in 3D-culture, 200 μl of the cell-containing collagen I was transferred into chamber slides and cultured in 10% FCS-containing medium for 24 h. These 3D-gels were incubated with anti-Thy1.1 antibody, and the Thy1.1 antigen was detected with DTAF-conjugated goat anti-rabbit secondary antibody. The polyclonal osteopontin antibody was kindly provided by A. Beshensky and J.G. Kleinman (Veterans Affairs Medical Center, Division of Nephrology, Milwaukee, WI, USA). The monoclonal antibody ox-7, which recognizes the Thy1.1 epitope, was purchased from Pharmingen (San Diego, CA, USA).

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DTAF, dichlorotriazinyl amino fluorescein; FCS, fetal calf serum; M, adenine, cytosine, or guanine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor

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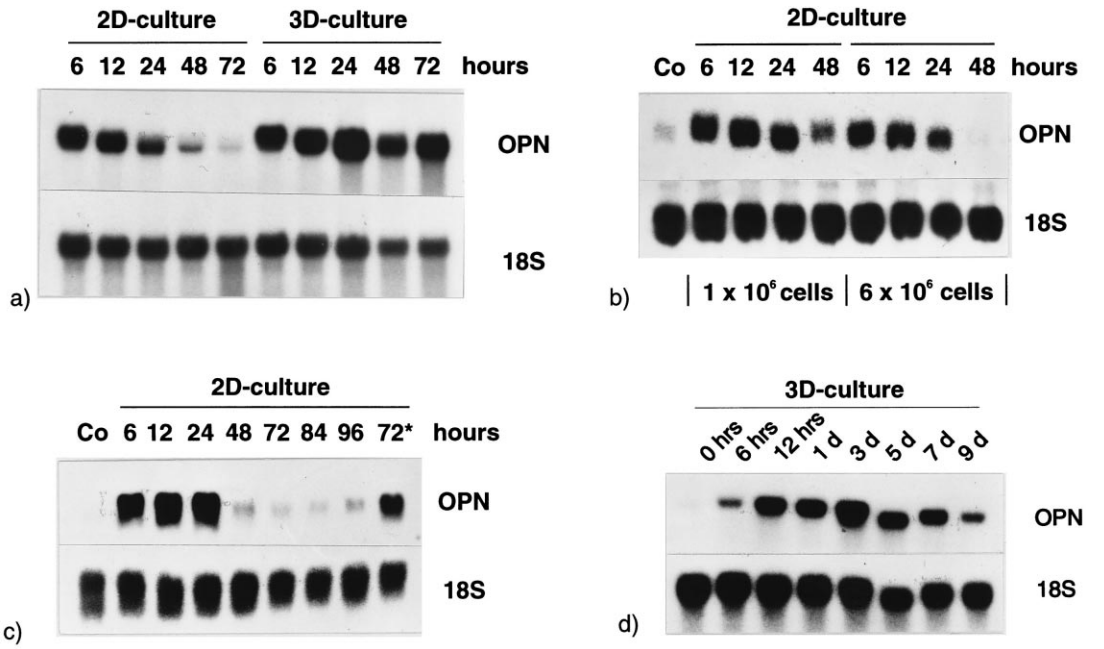

Fig. 1. Northern blot analysis of the osteopontin expression in rat mesangial cells. RNA was isolated at the time points indicated and hybridized with ^{32}P-labeled osteopontin and 18S cDNA probes (a–d). a: Cells were cultured in 2D- and 3D-cultures in the presence of 10% FCS. b: Confluent mesangial cells were trypsinized (Co) and plated at two different densities: 1×10^6 cells/dish (16 000 cells/cm^2) and 6×10^6 cells/dish (94 500 cells/cm^2). c: Confluent mesangial cells were trypsinized (Co) and 1×10^6 cells were plated per dish. (*) Medium was changed after 66 h, the cells were grown for another 6 h and harvested after a total of 72 h. d: Cells in 3D-culture were supplied with fresh 10% FCS-containing medium on days 1, 3, and 5.

2.5. Western blot analysis

Protein extraction: Cells grown in 2D-culture were harvested by scraping them off the dishes. From cells cultured in 3D-culture, gels were shock-frozen in liquid nitrogen, ground and extracted in lysis buffer. To purify secreted osteopontin from the medium, cells were grown to confluence in 10% FCS-containing DMEM and then incubated in serum-free DMEM for 48 h. The DMEM was collected and osteopontin was pelleted in the cold with 0.38% sodium citrate and 1.5% barium chloride. After centrifugation, the pellet was washed in 1.5% barium chloride and dissolved in 0.2 M sodium citrate (pH 6.8). Protein gels and immunostaining were performed as described previously [12]. Equal loading of cellular protein per lane was confirmed using Ponceau dye (Sigma Aldrich Chemical Co., Deisenhofen, Germany). Three series of independent experiments were done.

3. Results

3.1. Identification of differentially expressed genes

To investigate gene expression in proliferating and terminally differentiating rat mesangial cells, RNA was extracted from cells in 2D-culture 3 days after seeding and from cells subjected to 3D-culture after 6, 12, 24, 48, and 72 h. Differences in the gene expression patterns were analyzed by the differential display method. With the employed primer pair two differentially expressed mRNA species were identified. One of them exhibited low expression in 2D-culture and high expression in 3D-culture (data not shown). Sequence analysis of this clone showed 99.1% sequence homology over a stretch of 247 bp in the 3′ untranslated region to rat osteopontin [14]. Osteopontin expression was verified by Northern blot analysis. Osteopontin mRNA was initially high in 2D- and 3D-culturesY. In 2D-culture it was rapidly downregulated with lowest expression after 72 h when the cells became almost confluent. In 3D-culture, osteopontin mRNA levels remained unaltered at high expression level up to 72 h. (Fig. 1a).

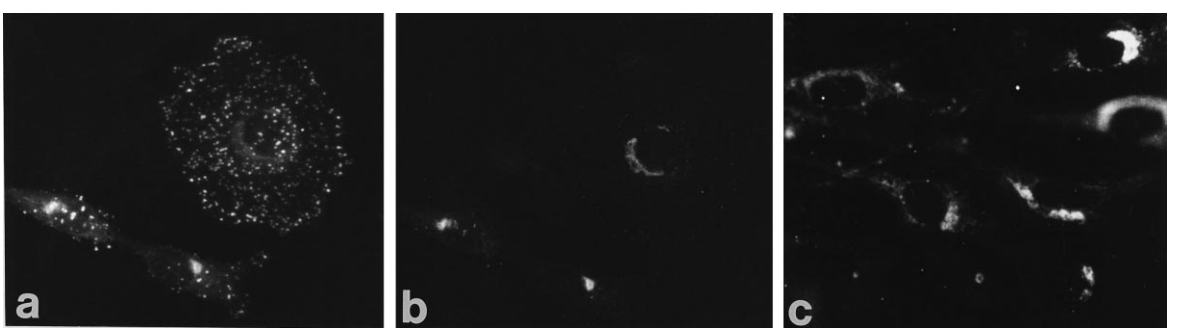

Fig. 2. Immunocytochemical detection of osteopontin in mesangial cells. Double-staining of the same cell with anti-Thy1.1-antibody specific for rat mesangial cells (a) and the osteopontin antibody in 2D-culture (b). Osteopontin staining of mesangial cells cultured in 3D-culture for 24 hours (c). Magnification 350×.

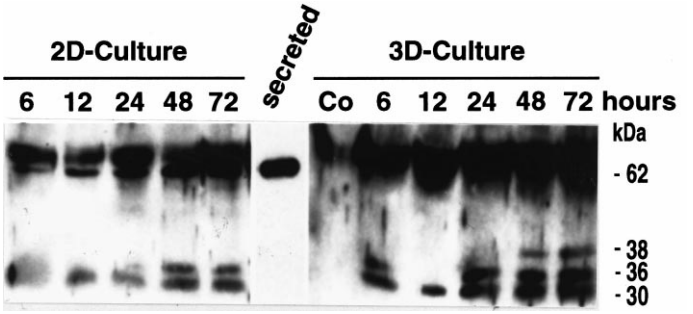

Fig. 3. Western blot analysis of osteopontin protein in 2D- and 3D-cultures. Protein was isolated from proliferating cells (2D-culture, lanes 1–5) and from terminally differentiating cells (lanes 7-11) at the time points indicated. Secreted osteopontin protein was isolated from conditioned, serum-free medium of confluent cells (2D-culture, lane 6). Cell-free collagen gels cultured in medium for 72 h served as control (Co).

3.2. Osteopontin expression in 2D-culture is induced by FCS irrespective of cell-cell contacts and proliferation rates

As shown in Fig. 1a, osteopontin mRNA levels declined with ongoing culturing time in proliferating cells. We investigated whether downregulation of osteopontin expression was linked to increasing numbers of cell-cell contacts. Mesangial cells were plated at low (10^6 cells/90 mm dish) and high (6×10^6 cells/90 mm dish corresponding to confluence) cell densities and osteopontin expression was assessed up to 48 h. Northern blot analysis showed that the osteopontin expression patterns were comparable, irrespective of the initial cell densities (Fig. 1b). Counting the cells revealed that the cell number had increased five-fold within 72 h when cells were plated at low density. There was a 1.3-fold increase when cells were plated at high density. These data indicated that osteopontin expression did not correlate with cellular proliferation.

To investigate whether osteopontin in 2D-culture was FCS-inducible, mesangial cells were plated at low density and harvested at the time points indicated (Fig. 1c). After 66 h, the culture medium was changed, the cells incubated for another 6 h with fresh medium containing 10% FCS, and harvested after a total of 72 h. Northern blot analysis showed that osteopontin expression could be reinduced by FCS (Fig. 1c).

3.3. Osteopontin expression in 3D-culture is independent of FCS

We investigated osteopontin expression in 3D-culture up to 9 days by changing the medium every second day (Fig. 1d) or once after 3 days (data not shown). Osteopontin mRNA levels were identical under both experimental conditions. Until the fifth day of 3D-culture, the levels were elevated, and they decreased after 7 and 9 days (Fig. 1d). Since this expression pattern was irrespective of medium changes, the data suggest that osteopontin expression was not FCS-inducible in 3D-culture.

3.4. Osteopontin protein expression in mesangial cells

Immunocytochemical analysis showed perinuclear localization of the osteopontin protein in 2D- (Fig. 2b) and in 3D-culture (Fig. 2c). The antigen Thy1.1 is a specific marker of rat mesangial cells [15]. By double-staining the cells with anti-Thy1.1 and osteopontin antibody, we could clearly show that osteopontin was synthesized in rat mesangial cells (Fig. 2a,b).

Western blot analysis showed three major osteopontin protein bands of about 34, 36, and 62 kDa in proliferating cells. While the 36 and 62 kDa bands remained at a comparable level from 6 to 72 h, the 34 kDa band increased after 48 and 72 h (Fig. 3, lanes 1–5). A 62 kDa osteopontin form was isolated from the medium of confluent mesangial cells (Fig. 3, lane 6). When the cells were subjected to 3D-culture, the discrete 62 kDa band was no longer detectable. Instead, protein labeling was observed as a bulk ranging from 50 kDa to 75 kDa, possibly due to posttranslational modifications. The 34 and 36 kDa bands remained clearly visible with increasing 36 kDa protein concentrations. A novel 38 kDa band appeared from 48 h 3D-culture onwards (Fig. 3, lanes 7–12).

4. Discussion

The aim of this study was to identify genes that were specifically induced or suppressed when the mesangial cell phenotype changed from a mitotically quiescent one in 3D-culture to the proliferative one in 2D-culture. Previous work has shown that mesangial cells and cells derived from different organ systems differentiated and maintained a phenotype closely related to the normal in vivo phenotype when subjected to 3D-culture [4,10,16–21]. Using the differential display strategy, we compared the gene expression patterns of both phenotypes and succeeded in identifying osteopontin as a differentially expressed mRNA transcript.

Osteopontin is a secreted extracellular matrix protein, which has been reported to be expressed in a variety of different cell types [22]. In healthy kidneys osteopontin is secreted into the tubule fluid [23], where it presumably inhibits renal stone formation [24,25]. Only very recently, osteopontin has also been shown to be expressed in rat mesangial cells [26]. Thus, besides the inhibition of stone formation, osteopontin might exert additional functions in the nephritic kidney.

We therefore analyzed the osteopontin expression pattern at the RNA and protein levels in more detail. Osteopontin mRNA levels were initially high when cells were plated in 2D-culture and declined gradually over days. Osteopontin expression was shown to be independent of the cell density and proliferation rate, but was induced and reinducible by FCS. In 3D-culture, osteopontin expression could not be reinduced by FCS addition. These data indicate that proliferating and quiescent cells respond in a differentiated manner to external stimuli.

Although not inducible by FCS, osteopontin mRNA levels in 3D-culture remained high for a prolonged period and declined only after 7–9 days. This suggests that either not yet identified stimuli of the surrounding matrix induced osteopontin mRNA and kept it at the elevated level, or that osteopon-

tin mRNA is stabilized in 3D-culture whereas non-stabilized RNA in 2D-culture decreased within 72 h.

Immunoblotting revealed four distinct osteopontin protein species of different sizes. Since Northern blot analysis showed a unique osteopontin mRNA band, we assume that the various protein bands were due to posttranslational modifications [22]. In 2D-culture, osteopontin RNA decreased while the protein concentration increased, suggesting that the protein was processed and continuously accumulated while the RNA was degraded. The distinct 62 kDa protein band, synthesized in 2D-culture, could not be visualized in 3D-culture. Instead, the protein bulk above stained stronger, which could be due to increasing posttranslational modifications or binding of the secreted osteopontin to other proteins. According to the time course of protein synthesis, we assume that the 62 kDa osteopontin protein is the synthesized – as well as the secreted – form, whereas the 34, 36, and 38 kDa species could be proteolytic cleavage products [22].

In conclusion, the presented data show that osteopontin is synthesized and secreted by rat mesangial cells in vitro. The mRNA expression pattern, the corresponding protein profile and the transcriptional regulation of osteopontin mRNA expression are clearly distinct in proliferating and terminally differentiating cells. This suggests that the deposition of the extracellular matrix protein osteopontin might be of functional importance for the modulation of the mesangial cell phenotype.

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