Creation of an In vivo cytosensor using engineered mesangial cells. Automatic sensing of glomerular inflammation controls transgene activity.

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Research Article

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Creation of an In Vivo Cytosensor Using Engineered Mesangial Cells

Automatic Sensing of Glomerular Inflammation Controls Transgene Activity

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Abstract

Automatic control over exogenous gene expression in response to the activity of disease is a crucial hurdle for gene transfer–based therapies. Towards achieving this goal, we created a "cytosensor" that perceives local inflammatory states and subsequently regulates foreign gene expression. α-Smooth muscle actin is known to be expressed in glomerular mesangial cells exclusively in pathologic situations. CArG box element, the crucial regulatory sequence of the α-smooth muscle actin promoter, was used as a sensor for glomerular inflammation. Rat mesangial cells were stably transfected with an expression plasmid that introduces a β-galactosidase gene under the control of CArG box elements. In vitro, the established cells expressed β-galactosidase exclusively after stimulation with serum. To examine whether the cells are able to automatically control transgene activity in vivo, serum-stimulated or unstimulated cells were transferred into normal rat glomeruli or glomeruli subjected to anti–Thy 1 glomerulonephritis. When stimulated cells were transferred into the normal glomeruli, β-galactosidase expression was switched off in vivo within 3 d. In contrast, when unstimulated cells were transferred into the nephritic glomeruli, transgene expression was substantially induced. These data indicate the feasibility of using the CArG box element as a molecular sensor for glomerular injury. In the context of advanced forms of gene therapy, this approach provides a novel concept for automatic regulation of local transgene expression where the transgene is required to be activated during inflammation and deactivated when the inflammation has subsided. (*J. Clin. Invest.* 1997. 100:1394–1399.) Key words: gene therapy • glomerulonephritis • mesangial cell • α-smooth muscle actin • CArG box element

Introduction

Ideal gene transfer systems should be competent for site-selective, long-term, and high levels of transgene expression. Previously, we reported an in vivo gene transfer approach that specifically targets the glomerulus of the kidney (1–3). This system uses the glomerular mesangial cell as a vector for gene delivery; that is, mesangial cells were propagated from isolated glomeruli, introduced with a foreign gene, and transferred back into the glomeruli via the renal circulation. The injected vector cells distribute throughout the kidney and accumulate in the glomeruli. More than 50% of isolated glomeruli express an exogenous gene product, and its sustained expression is observed for up to 4 wk (1). This mesangial cell vector system achieves efficient and site-specific delivery of a foreign gene into the glomerulus and allows for its sustained expression in vivo (4).

In some experimental and clinical settings, modifiable expression of transgenes is required for gene transfer approaches. For this purpose, the mesangial cell vector was combined with a tetracycline-dependent gene regulatory system (5), and a reversible on/off system was developed for in vivo control over glomerular transgene activity (6). In the gene transfer–based therapies of inflammatory disorders, however, tighter control of transgene depending on the activity of disease may be essential. Exogenous antiinflammatory molecules should be elaborated in response to inflammation, and their expression must be switched off after recovery from disease. Towards this goal, a potential strategy is to generate a local sensor that recognizes endogenous pathologic stimuli allowing for control of transgene activity. This would be achieved by the use of regulatory elements of a particular gene that is induced exclusively in pathologic conditions. By combining the mesangial cell vector with an α-smooth muscle actin promoter, the present study explored this idea, using glomerulonephritis as a disease model.

α-Smooth muscle actin, a muscle-specific actin isoform, is regarded to be a marker for activation and dedifferentiation of glomerular mesangial cells (7). In the normal glomerulus, expression of α-smooth muscle actin is absent or only faintly detectable (8–10). However, this actin isotype is markedly upregulated in mesangial cells during a wide range of experimental and human glomerular diseases, especially in mesangial proliferative glomerulonephritis (7–9, 11). This inducible property is possibly due to responsiveness of the α-smooth muscle actin promoter to endogenous pathogenic mediators released locally by platelets, infiltrating cells, and resident cells per se. The in vivo expression of α-smooth muscle actin is reversible, correlated with injury of the mesangium and/or activation of mesangial cells (8, 9, 11–13), and often precedes histopathological alterations (14, 15). The α-smooth muscle actin is thus a sensitive, general marker of glomerular injury, and its regula-

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tory element would be an ideal candidate as a molecular sensor for glomerular diseases.

The 5′-flanking region of the α-smooth muscle actin gene contains CC(A/T)6GG sequences (CArG box elements)[1] (16–19), the binding sites of a ubiquitous nuclear phosphoprotein, serum response factor (20). The CArG box motif, the core consensus sequence of serum response element (SRE), is crucial for serum-mediated induction of α-smooth muscle actin in various cell types (17–19). In rat mesangial cells, two CArG box motifs in the core promoter are necessary and sufficient for the induction of the α-smooth muscle actin gene (21). CArG elements are found in the regulatory regions of a number of growth factor–inducible genes (22–24) and are responsive to signals elicited by diverse pathogenic stimuli acting through different receptor types and signaling pathways (22, 25, 26). Based on these, the CArG box element would be useful for automatic upregulation of glomerular transgene activity in response to local, endogenous, inflammatory mediators. Using genetically engineered mesangial cells, this study aims to create an intraglomerular cytosensor that achieves automatic sensing of local inflammation and allows for subsequent control of transgene activity.

Methods

Establishment of sensing vectors. Rat mesangial cells were established from isolated renal glomeruli of an adult male Sprague-Dawley rat (27). A cell clone SM43 was established by a limiting dilution method (1) and identified as being of mesangial cell phenotype as described (27). Using a modified calcium phosphate coprecipitation method (28), SM43 mesangial cells were transfected with an expression plasmid pSRE-lacZ (29). This construct introduces (*a*) a bacterial β-galactosidase gene (*lacZ*) under the control of three copies of human c-*fos* SRE (5′-GGATGTGTCCATATTAGGACATCT-3′; the CArG box motif is underlined) adjacent to a minimal promoter derived from Rous sarcoma virus, and (*b*) a neomycin phosphotransferase gene (*neo*) driven by a thymidine kinase promoter. Stable transfectants were selected in the presence of neomycin analogue G418 (750 μg/ml; Sigma Chemical Co., St. Louis, MO), and sensing vectors MCAGZ1 and MCAGZ5 were established. MLTRZ mesangial cells that express *lacZ* under the control of a Moloney murine leukemia virus long terminal repeat (LTR) (1) were used as a control clone. Another control, MACTZ cells which express *lacZ* under the control of a β-actin promoter, was established by cotransfection of SM43 cells with pHβAPr-1-β-gal (30) and pRc/CMV (Invitrogen, San Diego, CA) which introduces *neo*.

Assessment of transgene activity. Confluent MCAGZ cells, MLTRZ cells, and MACTZ cells were cultured in the presence of a low (0.5%) or a high (15%) concentration of FCS for 72–96 h, and β-galactosidase activity was examined by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) assay (1, 6). In brief, cells were fixed in 0.5% glutaraldehyde, 2 mM $MgCl_2$, and 1.25 mM EGTA in PBS for 10 min at room temperature, and incubated at 37°C for 6 h in reaction buffer containing 1 mg/ml X-gal (Sigma Chemical Co.), 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, and 0.02% Nonidet P-40 in PBS (pH 7.4). To examine on/off kinetics of the β-galactosidase expression, FCS was depleted from cultures of serum-stimulated MCAGZ cells (15→0.5%) or added to serum-depleted cells (0.5→15%), and X-gal assay was performed before or 24, 48, and 72 h after the treatment.

To investigate whether the established sensing vectors are able to express the transgene in response to glomerular inflammation, cross-feeding studies were conducted in vitro. Conditioned media were prepared from isolated normal and nephritic rat glomeruli (31). To create the latter, anti–Thy 1 glomerulonephritis was induced by a monoclonal antibody 1-22-3 (32) in adult male Sprague-Dawley rats (body wt 200 g; two rats), as described previously (1). After 7 d, glomeruli were isolated (4.5×10^4; purity > 95%) and incubated for 24 h in 2 ml of culture media containing 1% FCS. Then, the media were collected, filtered, and stored at −80°C. Confluent MCAGZ5 cells were cultured in the presence of 0.5–2.5% FCS for 72 h and exposed to 1:1 diluted unconditioned medium or medium conditioned by normal or nephritic glomeruli. After 48 h, β-galactosidase expression was examined by X-gal assay.

In vivo control of glomerular transgene activity. We tested whether the established sensing vectors respond to conditions of local glomerular environment and subsequently modulate the transgene activity in vivo. Via the renal circulation, serum-stimulated or unstimulated MCAGZ5 cells were transferred into normal rat glomeruli (total of eight rats) or glomeruli of kidneys subjected to anti–Thy 1 glomerulonephritis (day 3; total of eight rats) (1). After 30 min and 3 d, glomeruli were isolated from both kidneys and used for X-gal assay to assess the β-galactosidase expression (6). To evaluate the transgene activity quantitatively, > 100 glomeruli were randomly selected, and percentages of X-gal–positive glomeruli were determined by light microscopy (1, 6).

Statistical analysis. Data are expressed as means±SE. Statistical analyses were performed using the nonparametric Mann-Whitney test. $P < 0.05$ was used to indicate a statistically significant difference.

Results

Inducible expression of a transgene in sensing vectors in response to serum stimulation. CArG box elements are known to be activated after exposure of cells to serum. The function of CArG elements introduced into mesangial cells was tested in vitro. Stably transfected mesangial cell clones (sensing vectors: MCAGZ1 and MCAGZ5 cells) that express *lacZ* under the control of CArG box elements were cultured in the presence of a low (0.5%) or a high (15%) concentration of FCS for

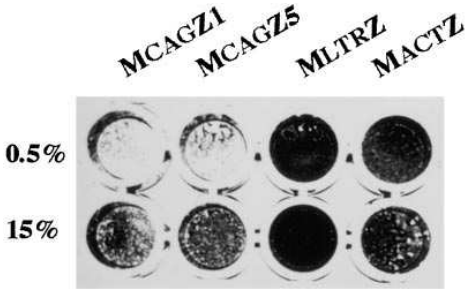

Figure 1. Inducible expression of a transgene in sensing vectors in response to serum stimulation. Sensing vectors MCAGZ1 and MCAGZ5 cells were established by transfection of mesangial cells with pSRE-lacZ which introduces *lacZ* under the control of three copies of CArG box elements. Control mesangial cell clones MLTRZ and MACTZ express *lacZ* under the control of a viral LTR and a β-actin promoter, respectively. Confluent MCAGZ cells, MLTRZ cells, and MACTZ cells were cultured in the presence of a low (0.5%) or a high (15%) concentration of FCS for 72–96 h, and β-galactosidase activity was examined by X-gal assay.

1. *Abbreviations used in this paper:* CArG box element, CC(A/T)6GG sequence; *lacZ*, bacterial β-galactosidase gene; LTR, long terminal repeat; *neo*, neomycin phosphotransferase gene; SRE, serum response element; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

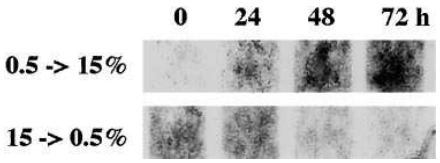

Figure 2. On/off kinetics of transgene activity in the sensing vectors. FCS was added to serum-starved MCAGZ5 cells (0.5→15%) or depleted from cultures of serum-stimulated cells (15→0.5%), and X-gal assay was performed before or 24, 48, and 72 h after the treatment.

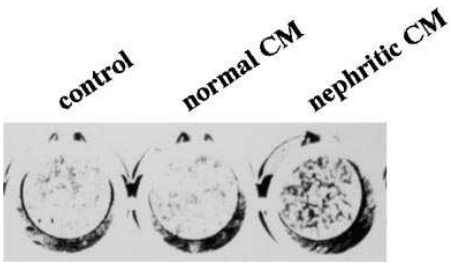

Figure 3. Induction of transgene expression in the sensing vector in vitro in response to mediators secreted from nephritic glomeruli. Conditioned media were prepared from isolated normal and nephritic rat glomeruli (anti–Thy 1 glomerulonephritis; day 7). Isolated glomeruli were incubated for 24 h in 2 ml of culture media containing 1% FCS, and the media were collected. Confluent MCAGZ5 cells were cultured in the presence of 0.5–2.5% FCS for 72 h and exposed to 1:1 diluted, unconditioned medium or medium conditioned by normal or nephritic glomeruli. After 48 h, β-galactosidase expression was examined by X-gal assay.

72–96 h, and β-galactosidase activity was examined by X-gal assay. As control clones, MLTRZ cells and MACTZ cells that express *lacZ* under the control of a viral LTR and a β-actin promoter, respectively, were used. Normally, rat mesangial cells express no endogenous β-galactosidase (1, 6). Under the low serum condition, the established sensing vectors expressed only limited levels of β-galactosidase activity. In contrast, serum-stimulated vector cells exhibited substantial upregulation of β-galactosidase (Fig. 1). This modifiable expression was observed neither in MLTRZ nor in MACTZ cells.

To examine on/off kinetics of the transgene activity, FCS was added to serum-starved MCAGZ5 cells (0.5→15%) or depleted from cultures of serum-stimulated cells (15→0.5%), and X-gal assay was performed before or 24, 48, and 72 h after the treatment. Serum-depleted MCAGZ5 cells showed only faint β-galactosidase activity. Stimulation of these cells by serum upregulated β-galactosidase within 24 h, which peaked at 48 h (Fig. 2, *top*). On the contrary, after serum depletion, the expression of β-galactosidase in serum-stimulated cells was substantially downregulated within 48 h (Fig. 2, *bottom*).

Induction of transgene expression in the sensing vectors in vitro in response to mediators released from nephritic glomeruli. To investigate whether the established sensing vectors are able to express the transgene in response to glomerular inflammation, cross-feeding studies were performed. Conditioned media were prepared from isolated normal rat glomeruli or inflamed glomeruli subjected to anti–Thy 1 glomerulonephritis (day 7). MCAGZ5 cells cultured under a low serum concentration were exposed for 48 h to diluted, unconditioned medium or medium conditioned by normal or nephritic glomeruli, and X-gal assay was performed. Conditioned media from normal glomeruli had no impact on the expression of β-galactosidase in MCAGZ5 cells. In contrast, the media conditioned by nephritic glomeruli significantly upregulated the β-galactosidase activity (Fig. 3). In mesangial cells transfected with pCRE-lacZ in which the CArG elements of pSRE-lacZ were replaced with cAMP response elements, β-galactosidase was induced by neither FCS nor the conditioned medium (data not shown).

On/off regulation of glomerular transgene activity in vivo in response to glomerular microenvironment. To investigate whether the established sensing vectors are able to respond to changes of local glomerular environment and subsequently to modulate the transgene activity in vivo, serum-stimulated cells ($MCAGZ5^+$) or unstimulated cells ($MCAGZ5^-$) were transferred into normal rat glomeruli or glomeruli of kidneys subjected to anti–Thy 1 glomerulonephritis (day 3). After 30 min and 3 d, glomeruli were isolated from both kidneys and used for X-gal assay to evaluate the transgene activity (Fig. 4, *A* and *B*). In the chimeric glomeruli of normal rats transferred with $MCAGZ5^+$ cells (Fig. 4 *A*, *top* and *B*, *left*), 68.1±9.0% (mean±SE, $n = 4$) of isolated glomeruli were positive for β-galactosidase at 30 min after the cell injection. However, after 3 d, this expression was switched off in response to normal glomerular environment (X-gal–positive glomeruli: 1.2±0.4%, $n = 4$, $P < 0.05$). In contrast, when $MCAGZ5^-$ cells were injected into nephritic rats (Fig. 4 *A*, *bottom* and *B*, *right*), β-galactosidase activity was detected only in 3.5±2.2% ($n = 4$) of isolated glomeruli at 30 min. However, the expression of β-galactosidase was substantially induced in vivo in response to the nephritic environment; i.e., after 3 d, 39.7±3.8% ($n = 4$) of isolated glomeruli were positive for X-gal staining. Throughout the experiments, the mean percentage of X-gal–positive glomeruli in noninjected right kidneys was 1.2±0.6%.

Discussion

Gene transfer–based therapies for inflammatory disorders require strict control over the transgene expression. Therapeutically relevant genes should be expressed exclusively in response to pathologic environment, and the expression must be turned off after recovery from disease. Towards achieving this end, one possible approach would be to create a system that perceives local inflammation. However, little attention has been paid to such sensing systems that allow for modifiable transgene expression. Using promoters of genes coding for acute-phase proteins, Varley and colleagues reported automatic induction of a transgene product in the liver in response to lipopolysaccharide challenge (33). For the treatment of local inflammation, however, this approach may have limitations because of (*a*) nonspecific induction of transgenes in response to various inflammatory conditions; (*b*) undesirable effects of transgene products on nontarget organs; or (*c*) limited levels of transgene products at the site of disease. A more sophisticated approach would be to generate a local sensor that recognizes endogenous pathologic stimuli and allows for local production of therapeutically-relevant molecules. By combining the mesangial cell vector system with the α-smooth muscle actin promoter, the present study addressed this idea, using glomerulonephritis as a disease model.

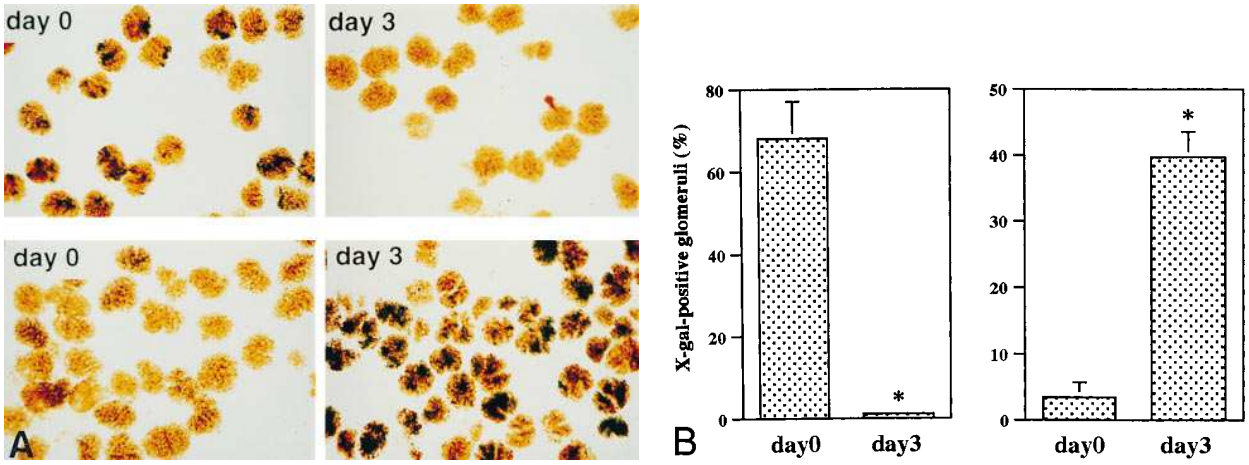

Figure 4. In vivo control of glomerular transgene activity. Via renal artery injection, serum-stimulated or unstimulated MCAGZ5 cells were transferred into normal rat glomeruli (*A*, *top*, and *B*, *left*) or glomeruli subjected to anti–Thy 1 glomerulonephritis (*A*, *bottom*, and *B*, *right*). After 30 min and 3 d, glomeruli were isolated from both kidneys and subjected to X-gal assay to evaluate the transgene expression. (*A*) Light microscopy. (*B*) Quantitative analysis. More than 100 glomeruli were randomly selected, and percentages of X-gal–positive glomeruli were determined. Data are expressed as means±SE. Asterisks indicate statistically significant differences ($P < 0.05$).

Recent investigation on phenotypic change of glomerular mesangial cells has shed light on α-smooth muscle actin as a marker of mesangial cell activation (7). In contrast to vascular smooth muscle cells and pericytes, this actin isotype is absent in normal mesangial cells but induced exclusively under pathologic situations. In rat mesangial cells, a core sequence of the α-smooth muscle actin promoter from positions −122 to +1 is essential for serum induction of α-smooth muscle actin gene (21). This sequence contains two highly conserved CArG box motifs that alone confer maximum levels of serum inducibility on a heterologous minimal promoter. Deletion of either sequence reduces the target gene transcription, suggesting that both CArG box elements are required and sufficient for efficient transcription (21). This has led us to use the CArG box motif as a sensor for glomerular injury. Using the established sensing vectors, the present study demonstrated that (*a*) activity of CArG box elements is depressed in serum-depleted, inactivated mesangial cells; (*b*) CArG box element is active in serum-stimulated mesangial cells that mimic activated, in vivo mesangial cells; and (*c*) the CArG box activity in mesangial cells is switched off within the normal glomerular environment but turned on in the nephritic glomeruli. These data elucidated that the CArG box element linked to a minimal promoter is useful as a sensor of glomerular injury. The use of mesangial cells may be required since, in pathologic situations, α-smooth muscle actin is expressed in mesangial cells but not in glomerular epithelial and endothelial cells.

In anti–Thy 1 glomerulonephritis, several endogenous mediators may be implicated as triggers of CArG box elements. PDGF is a possible candidate. Glomerular PDGF is upregulated in this nephritis model (34), and PDGF induces activation of CArG box elements in cultured rat mesangial cells (35). Another potential candidate could be TGF-β1. TGF-β1 is known to be expressed in mesangial cells during the course of this experimental disease (36). A preliminary result has shown that introduction of a TGF-β1 gene into the glomerulus induced α-smooth muscle actin expression in vivo (37). Similarly, angiotensin II might be involved as a stimulator because systemic infusion of angiotensin II induces α-smooth muscle actin in mesangial cells (15). Other vasoactive mediators such as arginine vasopressin and endothelin, both of which induce activation of CArG box element in mesangial cells, could also be implicated (38, 39). It is worthwhile to note that all of these have been regarded as potential mediators of glomerular injury (40–43).

Based on the fact that CArG box element is responsive to various pathogenic peptide factors, this sensing system would be applied for a diverse range of cytokine-mediated glomerular injury. Furthermore, CArG box element could also be useful for sensing other pathologic situations including reactive oxygen–mediated or hemodynamic stress–associated glomerular damage (44–47). Since expression of α-smooth muscle actin occurs in the early stage of diseases (12), occasionally preceding histopathological alterations (14), early, sensitive detection of glomerular damage may be feasible. CArG box element is possibly useful to detect not only mesangial injury but also mesangial "perturbation" in association with minimal change disease, amyloidosis, diabetic glomerulopathy, or systemic hypertension where histopathological changes are dormant (8, 9, 11).

In advanced forms of gene therapy, several properties are required for the sensing systems. The activity of regulatory elements should be silent in the normal state but substantially activated in response to a wide range of endogenous, pathogenic triggers. After recovery from disease, the transgene expression should be immediately turned off. The promoter activity must be strong, and the transgene regulation is expected to be rapid, reversible, and hopefully stepwise depending on the activity of diseases. Using CArG box element, this study demonstrated that the established sensing system meets some of these demands. Other inflammation-responsive promoters could also be useful for this purpose. For example, the 12-*O*-tetradecanoylphorbol 13-acetate response element (TRE) and the NF-κB binding site are activated in nephritic glomeruli (48, 49). TRE and the κB site are located in regulatory regions of

various pathogenic genes and are activated in response to pathologic mediators including cytokines and growth factors (50–51). This implies potential utility of these regulatory elements as alternative genetic sensors for glomerular injury.

In summary, using the mesangial cell vector combined with CArG box elements, automatic regulation of transgene expression in response to glomerular inflammation has been achieved. To our knowledge, this is the first establishment of an in vivo sensing system that allows for automatic on/off control of transgene activity in response to local inflammatory states.

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