行政院國家科學委員會專題研究計畫 成果報告

以新合成 的TGF-beta 拮抗劑探討TGF-beta對腎臟纖維化的角色(3/3)

研究成果報告(完整版)

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行政院國家科學委員會補助專題研究計畫成果報告

以新合成的 TGF-beta 拮抗劑探討 TGF-beta 對腎臟纖維化的角色

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中文摘要:

如何減少慢性腎病惡化是目前腎臟界研究的一個重要課題。已知 TGF-beta 在腎臟纖維化的致病過程中扮演重要的角色。在此三年計畫中，第一年及第二年我們利用人工合成的 TGF-beta 拮抗劑觀察其在細胞和大鼠腎小球腎炎模式的療效。在細胞方面，TGF-beta 拮抗劑的確可以截斷 TGF-beta 的訊息傳遞及減少 TGF-beta 所引起的細胞外基質增生。但在腎小球腎炎的治療方面，TGF-beta 拮抗劑並沒有明顯的保護效果。

因此在計畫的第二年及第三年，我們利用 ALK5 inhibitor SB431542 取代 TGF-beta 拮抗劑，觀察其在細胞和大鼠腎小球腎炎模式的作用。結果顯示 SB431542 的確可以有效的截斷 TGF-beta 的訊息傳遞及減少 TGF-beta 所引起的細胞外基質增生。在腎小球腎炎的治療方面，SB431542 可以減少腎絲球中基質基因的表現及減少腎小球腎炎嚴重程度。但在尿液中蛋白的分泌上卻沒有看到確定的效果。

關鍵詞: 腎臟纖維化，TGF-beta 拮抗劑，腎小球腎炎

英文摘要

Abstract:

In the fist and second year of this three-year project, first and second year, we observed a protective effect of TGF-β1 antagonist in cells stimulated by TGF-beta, and studied further the effect of TGF-β1 antagonist in a rat model of glomerulonephritis. In rat mesangial cell (RMC), we found the antagonist can block TGF-beta-induced signal transduction and reduce extracellular matrix production. In rats, we used anti-CD90 (anti-Thy1.1) antibody to induce glomerulonephritis, and treated the nephritic rats with the antagonists. We found no significant protective effect in rats receiving the antagonist treatment. Therefore, in the second and the third year, we replaced the antagonist with an ALK5 inhibitor SB431542 to study the effect in blocking TGF-beta-induced signals on RMC, NRK-52E and mouse podocytes. The results showed SB431542 had significant effects in blocking TGF-beta-induced signals and in reducing connective tissue growth factor(CTGF) and plasminogen activator inhibitor1(PAI-1) gene expression. Besides, we also studied the possible protective effect of SB431542 rats with anti-Thy1.1 nephritis. The preliminary data indicated that SB431542 had potential protective effects in reducing matrix gene expression and attenuating the severity of histopathology. However, there was conflicting results in the urine protein excretion.

Key words: renal fibrosis, TGF-beta antagonist, glomerulonephritis

Introduction:

The most prominent pathological change seen in progressive renal disease regardless of primary etiology is a process by which normal glomerular structure is replaced by accumulated deposits of extracellular matrix (Rennke et al, 1994). According to previous reports, we knew TGF-beta plays an important role on the development and progression of renal fibrosis (Bottinger & Bitzer, 2002). Therefore, there were a variety of physiological pharmacological and molecular approaches have been used to study the potential therapeutic effects of blocking TGF beta and/or its downstream signals.

In this project, we use two kinds of compounds to study the effect of blocking TGF-beta. One is a synthetic TGF-beta1 pentacosapeptide(β1(41-65)), whose amino acide sequences correspond to the 41 th to 65th amino acid residues of TGF-beta can competitively antagonize TGF-beta1 action(Huang et al, 1997). The other one is TGF-beta receptor ALK5 kinase inhibitor, SB431542. We studied the effect of these two compounds in TGF-beta signals transduction, extracellular gene expression, and the severity of rat anti-Thy1 glomerulonephritis.

Result:

Part 1.Treatment by TGF-beta antagonist

(1) Results of cultured RMC

RMC were treated with TGF-beta (1ng/ml for western blot and 5ng/ml for Northern blot), TGF-beta antagonist (A7.5, 15, 30μM) and polyethylene glycol (PEG7.5, 15, 30μM) as an internal control. The protein and mRNA were extracted to study. The experiment results are shown as follow.

Fig.1

RMC were treated with TGF-beta 1ng/ml and different doses of antagonist to observe the expression of p-Smad2 protein.

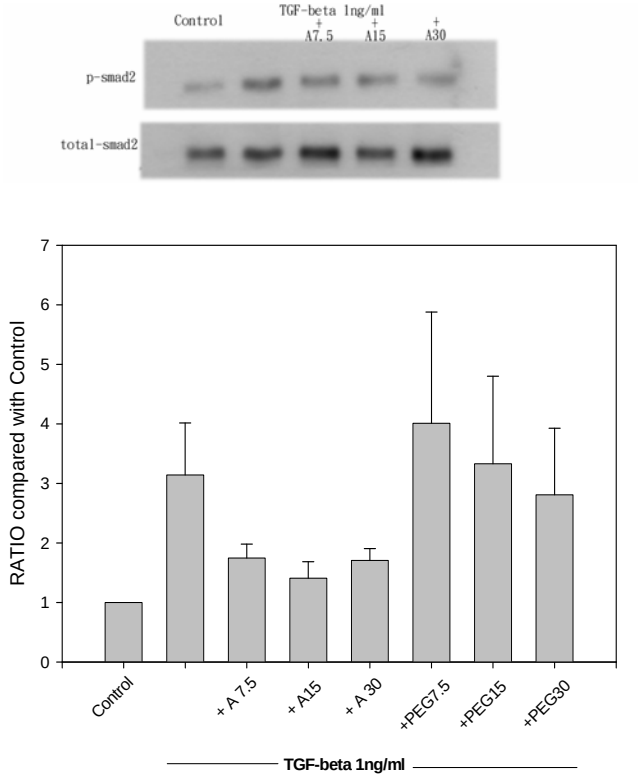


Fig.2

Phospho-Smad2 induced by different concentrations of TGF-beta was decreased by antagonist at 30μM.

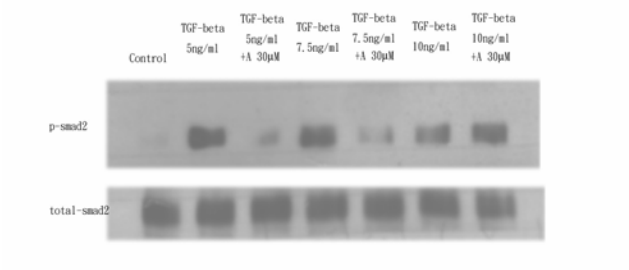
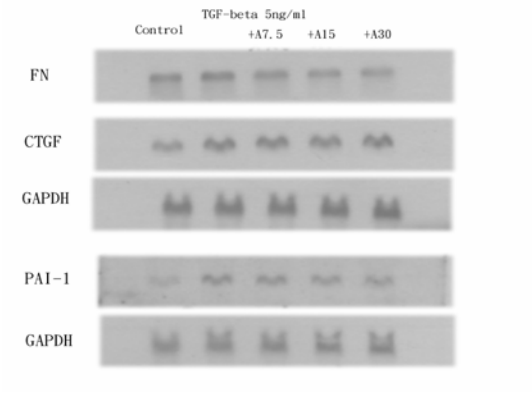


Fig.3

The expression of TGF-beta induced FN, PAI-1 mRNAs were reduced by different concentrations of the antagonist.



(2)Rat Thy1.1 model

Male Wistar Rats around 200g were

induced glomerulonephritis with mouse anti-CD90 (anti-Thy1.1) antibody (0.125ml/rat) (CL005A, Cedarlane). After antibody injection, rats were treated with control drug PEG 100μl or TGF beta antagonist 1mM 100μl intravenously/day.

Table1. The urine protein excretions are listed as below.

	1st day urine protein (mg)	3rd day urine protein (mg)	5th day urine protein (mg)
Normal control	4.684	4.336	4.744
Rat Thy1 + PEG	8.273	59.745	51.349
Rat Thy1 + PEG	5.512	95.776	49.674
Rat Thy1 + antagonist	18.855	206.192	171.583
Rat Thy1 + antagonist	5.294	104.718	41.924

There was no significant difference in urine protein excretion between control and antagonist-treated rats.

There was also no difference in histopathology (H&E stain) between Thy1+PEG rats and Thy1+antagonist rats.

Immunohistochemical stains were performed using ant-ED-1 antibody to show the monocytes/macrophages and anti-alpha-smooth muscle actin antibody to show the activated mesangial cells. After counting the positive cells in 20 glomeruli, there was no difference in the expression of ED-1 and alpha-smooth muscle actin positive cells between PEG and antagonist treated rats.

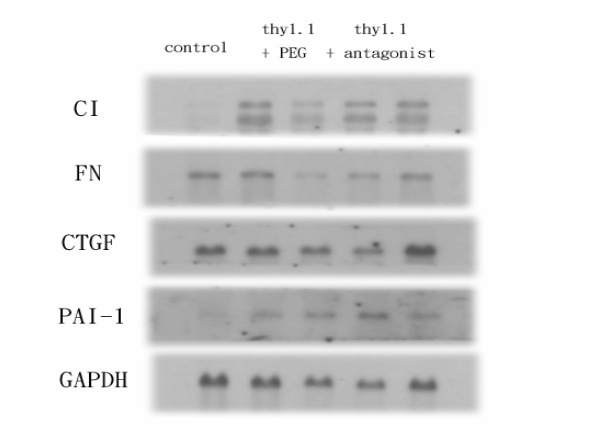


Fig.4
The expression of extracellular matrix mRNA of rats.

Part 2: Treatment by SB431542

(1) Results of cultured RMC :

RMC were treated with TGF-beta 1ng/ml and SB431542 0.5, 1, 5μM for 15min to observe he expression of p-Smad2 protein. The result showed SB431542 can inhibit the TGF-beta-induced p-Smad2 protein expression does-dependently. In the SB431542 5μM treatment, the p-smad2 expression was reduced to the same degree as the control (Fig.1-1). And the inhibitory effect of SB431542 5μM in TGF-beta 1ng/ml stimulation persisted for at least 8h (Fig.1-2).

Fig.1-1

RMC were treated with TGF-beta 1ng/ml and different doses of SB431542 to observe the expression of p-Smad2 protein.

*Students' t test $p<0.05$, compared with TGF-beta+DMSO.

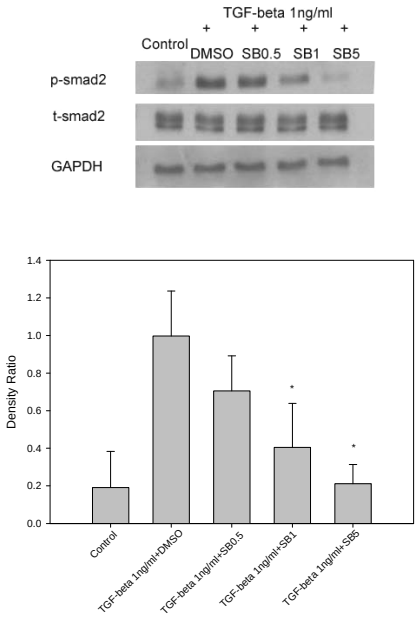
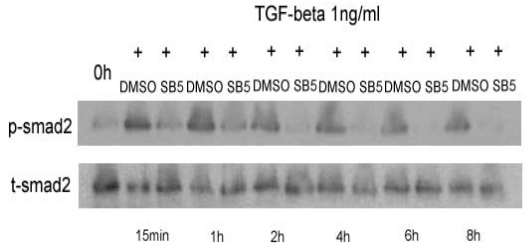


Fig1-2

RMC were treated with TGF-beta 1ng/ml and SB431542 5μM at different time points to observe the changes in the expression of p-Smad2 protein.

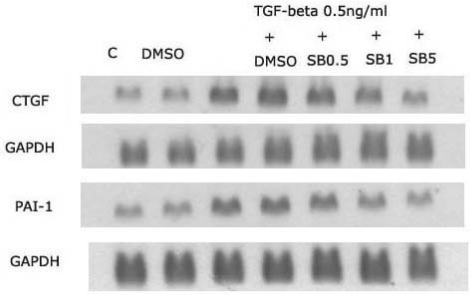


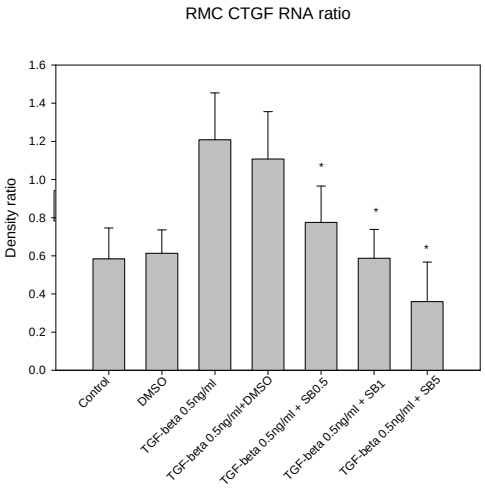
To study matrix gene mRNA expression, RMC were treated with TGF-beta 0.5ng/ml and SB431542 0.5, 1, 5μM for 4h to observe the CTGF and PAI-1 mRNA expression. The results showed SB431542 could reduce TGF-beta-stimulated mRNA expression dose-dependently. (Fig.2-1). And the inhibitory effect of SB431542 5μM in TGF-beta 0.5ng/ml persisted for 24h (Fig.2-2)

Fig.2-1

The expression of TGF-beta induced CTGF and PAI-1 mRNAs were reduced by different concentrations of SB431542 at 4h-treatment.

*Students' t test $p<0.05$, compared with TGF-beta+DMSO.





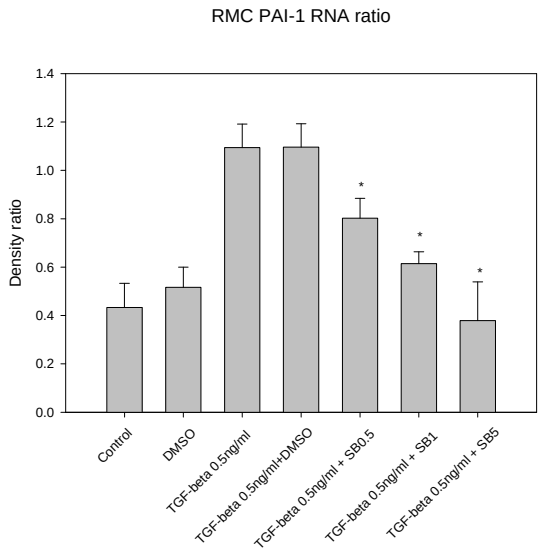
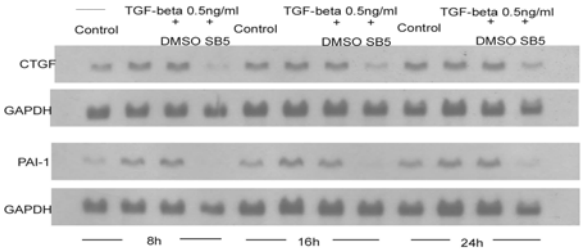


Fig.2-2
RMC were treated with TGF-beta 0.5ng/ml and SB431542 5μM at different time points to observe the changes in CTGF and PAI-1 mRNA expression.



(2) Results in cultured NRK-52E

NRK-52E were treated with TGF-beta 0.5ng/ml and SB431542 0.05, 0.1, 0.5, 1μM for 15min to observe the expression of p-Smad2 protein. The results showed that SB431542 significantly inhibited TGF-beta-induced p-Smad2 protein expression. (Fig.3-1) And the inhibitory effect of SB431542 0.5μM in TGF-beta 0.5ng/ml stimulation persisted for 6h (Fig.3-2).

Cells were treated with TGF-beta 10ng/ml for 1d. TGF-beta induced alpha-smooth muscle actin (α-SMA) in NRK-52E cells, which could be reversed by SB431542 treatment. (Fig.4)

Fig.3-1
NRK-52E were treated with TGF-beta 0.5ng/ml and different doses of SB431542 to observe the changes in the expression of p-Smad2 protein.

*Students' t test $p<0.05$, compared with TGF-beta + DMSO.

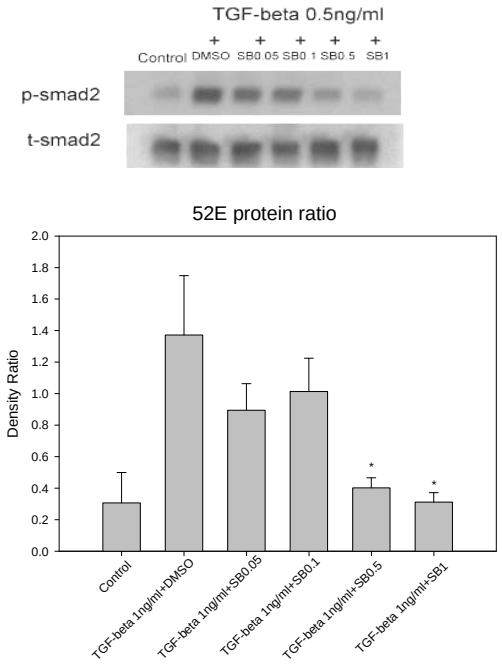


Fig.3-2
NRK-52E were treated with TGF-beta 0.5ng/ml and SB431542 0.5μM at different time points to observe the changes in the expression of p-Smad2 protein.

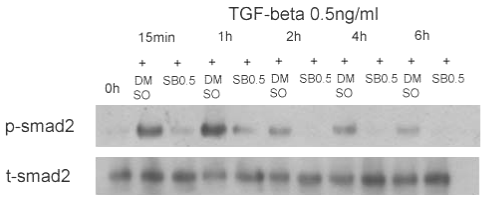
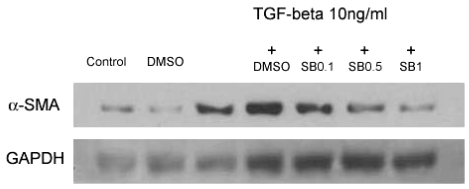
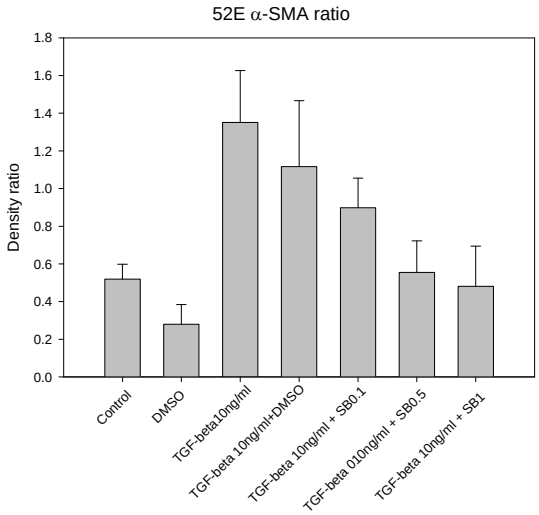


Fig.4
NRK-52E were treated with TGF-beta 10ng/ml and different doses of SB431542 to observe the effects on the expression of α-smooth muscle actin.



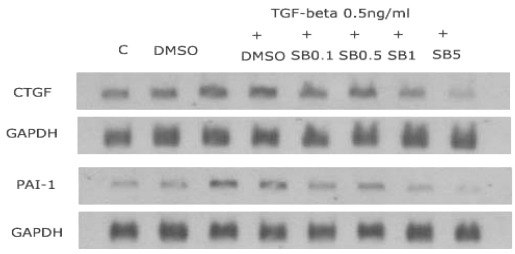


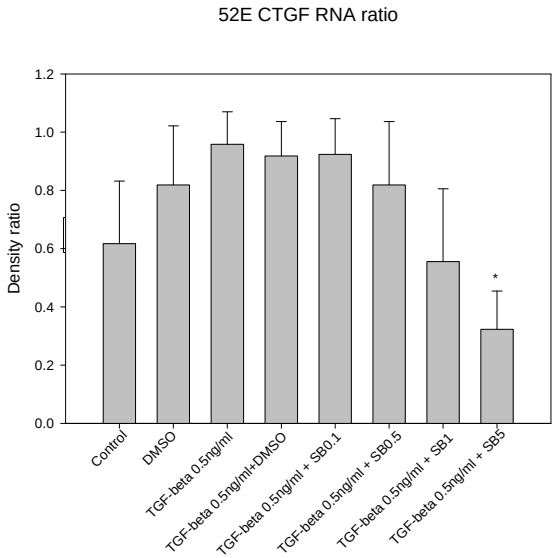
NRK-52E were treated with TGF-beta 0.5ng/ml and SB431542 0.5, 1, 5μM for 4h to observe the effects on CTGF and PAI-1 mRNA expression. The results showed TGF-beta increased the expression of CTGF and PAI-1 mRNAs, which could be inhibited by SB431542 treatment. (Fig.5)

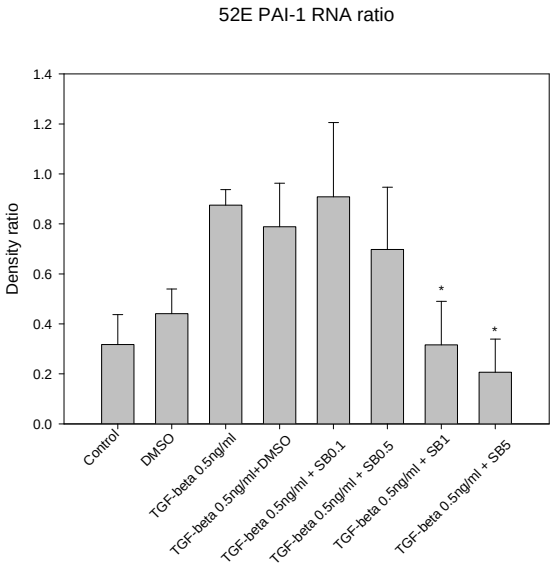
Fig.5

The expression of TGF-beta 0.5ng/ml induced CTGF and PAI-1 mRNAs were reduced by different concentrations of SB431542 at 4h-treatment.

*Students' t test $p<0.05$, compared with TGF-beta + DMSO.







(3) Results in cultured mouse podocytes.

Podocytes were treated with TGF-beta 0.5ng/ml and SB431542 0.05, 0.1, 0.5, 1μM for 15min to observe the effects on the expression of p-Smad2 protein. The results showed SB431542 inhibited TGF-beta-induced p-Smad2 protein expression. (Fig.6)

Fig.6

Podocytes were treated with TGF-beta 0.5ng/ml and different doses of SB431542 to observe the changes in the expression of p-Smad2 protein.

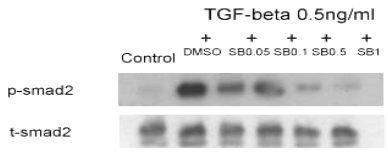
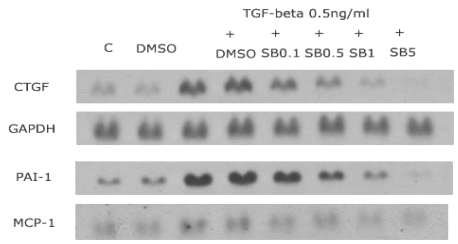


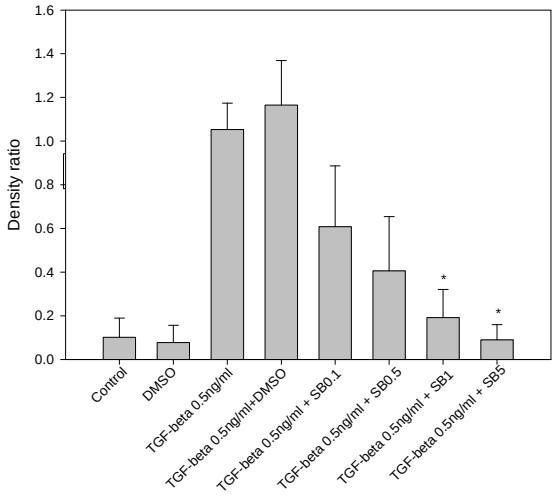
Fig.7

The expression of TGF-beta 0.5ng/ml induced CTGF and PAI-1 mRNAs were reduced by different concentrations of SB431542 at 4h-treatment.

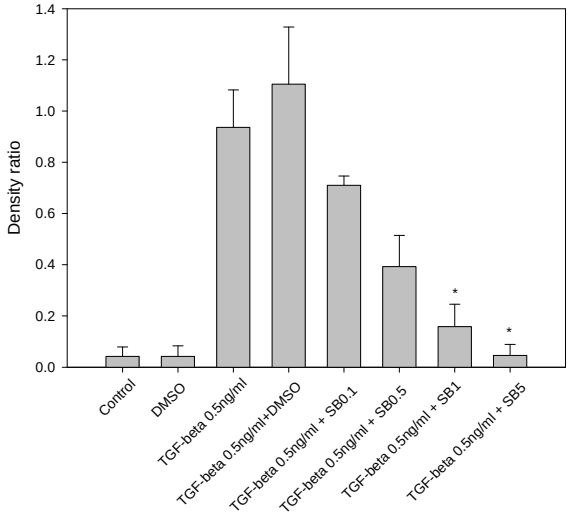
*Students' t test $p<0.05$, compared with TGF-beta + DMSO.



podocyte CTGF RNA ratio



Podocyte PAI-1 RNA ratio



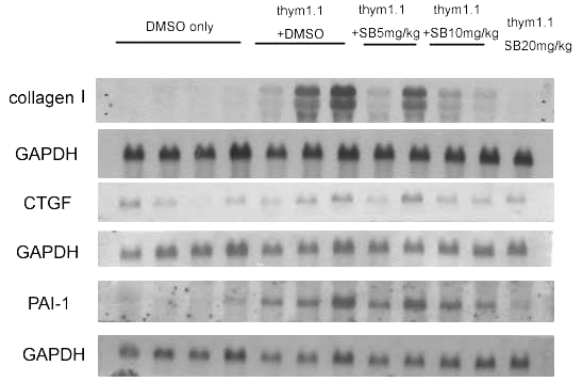
(4)Rat Thy.1.1 model

Male Wistar Rats around 200g were used. Rats were divided into 5 groups. Group1: rats were treated with DMSO only. Group2: rats were induced anti-Thy1.1 glomerulonephritis with mouse anti-CD90 (anti-Thy1.1) antibody. After antibody injection, rats were treated with control drug DMSO /ml /kg /day ip for 5day. Group3, 4, 5: nephritic rats were treated with SB431542 5mg, 10mg, 20mg/ml/kg/day ip. respectively for 5day.

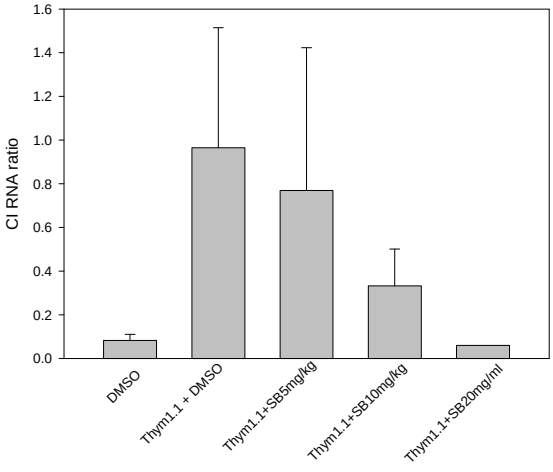
The glomerular RNA were extracted to observe matrix gene expression. The results showed that SB431542 treatment reduced collagen gene expression at a dose higher than 10mg/kg. (Fig.8) But urine excretion was not reduced for sure.

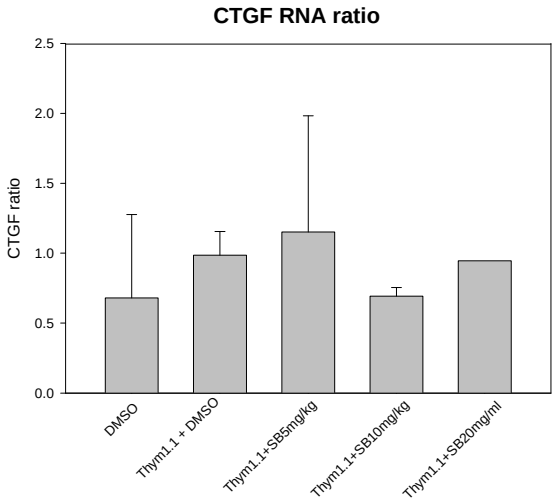
Fig.8

The expression of matrix gene in rats receiving different doses of ALK5 inhibitor.



Collagen I RNA ratio





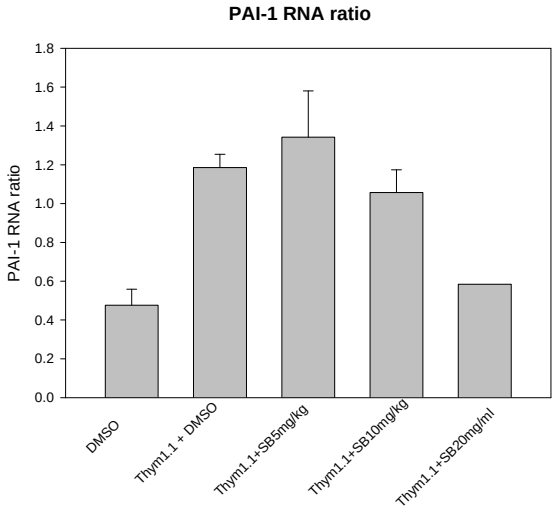
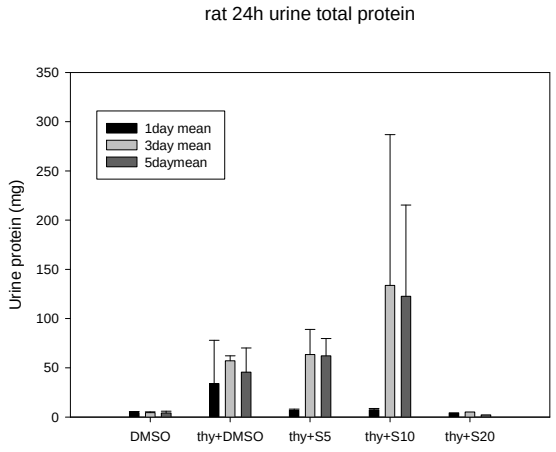


Fig. 9
The protein excretion in rat 24h urine.



Discussion:

In RMC, the antagonist is capable of blocking TGF-beta induced signals, and attenuates matrix gene expression. But these effects tend to diminish because the competitive nature of this antagonist.

In the animal model, we didn't observe a therapeutic effect of the TGF-beta antagonist. This result might be due to insufficient dose of the antagonist, or improper way of giving the antagonist.

In RMC, 52E and podocyte, SB431542 consistently blocks TGF-beta downstream signals, as well as TGF-beta-stimulated CTGF and PAI-1 gene expression.

In the anti-thy1.1 model, SB431542 inhibited collagen I gene expression and attenuated the severity of glomerulosclerosis but there was no appreciable effect in urine protein excretion. These results still need to be confirmed by increasing the number of experiments in the future.

Conclusion:

Comparing the effects of the synthetic TGF-beta antagonist and SB431542 in cell aspect and anti-thy1 model, we found ALK5 inhibitor SB431542 seems to have higher potential protective effects.

計畫結果自評:

In past three years, we found TGF-beta antagonist had protective effect in cells, but not showed the protection in anti-Thy1.1 model. Then we replaced it with ALK5 inhibitor SB431542. It seems to have therapeutic effect both in cell and anti-thy1 model. Although the results still need to be confirmed by increasing the number of experiments, we hope the results can contribute to the therapy of renal disease.

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partial agonists. Journal of Biological Chemistry. 272(43):27155-9, 1997

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