# Role of IP-10 and its receptor CXCR3 in development and progress fibrosis in kidney



# ケモカインを介した
# 腎発生ならびに再生機序とその制御

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# はしがき

現在まで腎障害にはたすケモカインおよびその受容体の役割と治療への応用について検討してきた. すでに interleukin-8 (IL-8)に関しては急性期腎炎にはたす役割を明らかにした(Kidney Int, 1994, J Exp Med, 1994). さらに monocyte chemoattractant protein-1 (MCP-1)について, ヒトループス腎炎, IgA 腎症および現在我国における末期腎不全の第 1 位の原因となった糖尿病腎症の発症・進展における MCP-1 の関与を明らかにした (Kidney Int 1996, 2000). さらにヒト腎疾患において免疫学的ならびに非免疫学的機序の如何を問わず尿中 MCP-1 は腎疾患の進行とともに増加した. 組織学的には間質線維化といった病態特異的に深く関与することから, MCP-1 は病因を問わず共通の進展因子であることを報告した (J Leukoc Biol 1998, Kidney Int 2000). 加えて進行性腎炎モデルに対し抗 MCP-1 抗体を投与したところ腎機能保持効果, 腎硬化性病変・間質線維化改善を確認した (FASEB J, 1996). さらに MCP-1 とその受容体 CCR2 を治療標的分子とすることにより尿細管間質障害である急性尿細管壊死や間質線維化の制御が可能であることを示してきた. すなわち MCP-1 変異体を用いた遺伝子治療(J Am Soc Nephrol 2003), CCR2 阻害薬(J Am Soc Nephrol 2003)および CCR2 欠損マウス(J Am Soc Nephrol 2003)を用いることによりその有用性を示してきた. 加えて MCP-1/CCR2 に代表されるケモカインの発現ならびに受容体のシグナル伝達に重要な p38 mitogen activated protein kinase(MAPK)阻害薬を用いることによりケモカインの抑制を介して進行性ループス腎炎(J Am Soc Nephrol 2003)ならびに糸球体硬化・間質線維化(J AM Soc Nephrol 2000, Am J Kidney Dis 2001)の進展抑制効果を示してきた. 以上よりケモカインは腎疾患の発症・進展に重要であり, 診断・病勢判断といった臨床応用に加え, 尿細管間質障害の抑制から腎疾患の進展阻止にむけた重要な治療標的分子となりうることが期待される.

一方, 進行性腎疾患の治療を考える上で, 上記に述べた進展阻止のみならず, 障害細胞の再生／修復は非常に魅力的で究極的な治療法である. 一般に腎に限らず発生／形成過程でみられる機序は臓器再生／修復との類似点が多い. この再生／修復と発生／形成過程では細胞増殖, 細胞遊走, 機能分化誘導ならびに細胞外基質代謝が重要と考えられている.しかしながらこれまでケモカインによる腎発生／形成ならびに腎再生／修復機序における役割は目下のところ十分に理解されていない. CXC ケモカインファミリーに属する interferon

inducible protein (IP)-10 はヒト胎児腎において発現が報告されており大変興味がもたれる．さらに IP-10 は細胞遊走のみならず腎固有細胞の増殖作用を示す．これは再生／修復と発生／形成過程において IP-10 がその作用を介して重要な役割をはたしていることを示唆する．さらに尿細管間質病変は腎疾患の病因を問わない予後規定因子であり，その治療は予後改善に重要な課題である．

そこで腎発生過程ことに尿細管上皮細胞で発現が確認されている IP-10/CXCR3 に着目し，腎発生ならびに腎進行性線維化への関与とその制御による抗線維化の治療戦略構築にむけた研究を行った．

# 研究組織

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# 研究成果による工業所有権の出願・取得状況

特になし

# 研究成果

ヒト進行性腎間質線維化は腎不全に至る, 病因を問わない共通の進展機序である. 従って腎間質線維化機構を明らかにすることは腎疾患の治療を考える上で極めて重要な意味を持ち, 予後の改善に繋がる可能性がある. 腎局所へ浸潤した炎症細胞, 免疫担当細胞はその活性化および腎固有細胞との相互連関を介して腎疾患の発症・進展に重要な役割をはたす. 従ってこれら細胞の浸潤・活性化機構の解明は腎疾患進展機序ならびに治療を考えるうえで重要な意味をもつ. 本研究では細胞の遊走・活性化能を有するケモカイン Interferon-inducible protein-10(IP-10)/CXCL10 およびその受容体 CXCR3 に焦点をあて, 発生ならびに進行性腎間質線維化機構への関与ならびにその制御による抗線維化の治療戦略構築にむけた研究を行った. 以下にその成果を報告する.

1) IP-10/CXCR3 の発生ならびに腎障害時の再生/修復への関与
2) IP-10/CXCR3 の進行性腎線維化過程における意義

1)　IP-10/CXCR3 の発生ならびに腎障害時の再生/修復への関与

a) Interferon-inducible protein-10(IP-10)/CXCL10 およびその受容体 CXCR3 を介す

る腎障害時の再生ならびに発生への関与について

はじめに腎発生における IP-10 とその受容体 CXCR3 の発現をマウス胎児腎において検討した. その結果, E13.5 において IP-10 ならび CXCR3 はマウス胎児期において糸球体原基ならびに間質内に発現がみられることが判明した. マウス腎は生後 1 週間かけて成熟すると考えられている. この期間, 成熟過程にあるのが nephrogenic area と呼ばれる皮質である. 生後もこの nephrogenic area に IP-10/CXCR3 の発現が認められる. しかしながら, 一旦, マウス腎が成熟するとほとんど IP-10/CXCR3 発現がみられなくなった. この観察から現時点ではその役割は不明な点が多いものの, IP-10/CXCR3 は腎発生になんらかの影響をはたしているものと推測される.

一方, 一旦 IP-10/CXCR3 発現が消失したマウス成熟腎を用いて虚血再環流障害モデルを作成したところ, IP-10 ならびに CXCR3 ともに間質浸潤細胞ならびに尿細管上皮細胞に発現を認めた. この新たに発現がみられる IP-10/CXCR3 の役割を検討する目的で, 虚血再環流障害モデル作成時に抗 IP-10 中和抗体投与を行った. その結果, 抗 IP-10 中和抗体投与群において, 対照群と比較して再環流後 4 日で尿細管上皮細胞の再生が増加することが判明した（図 1）. さらにこの虚血再環流モデルにおいて, 尿細管上皮細胞増殖の亢進が Ki67 陽性細胞数増加より示された（図 2）. 一方, このモデルの特徴である尿細管壊死は抗 IP-10 中和抗体投与による差異はみられなかった.

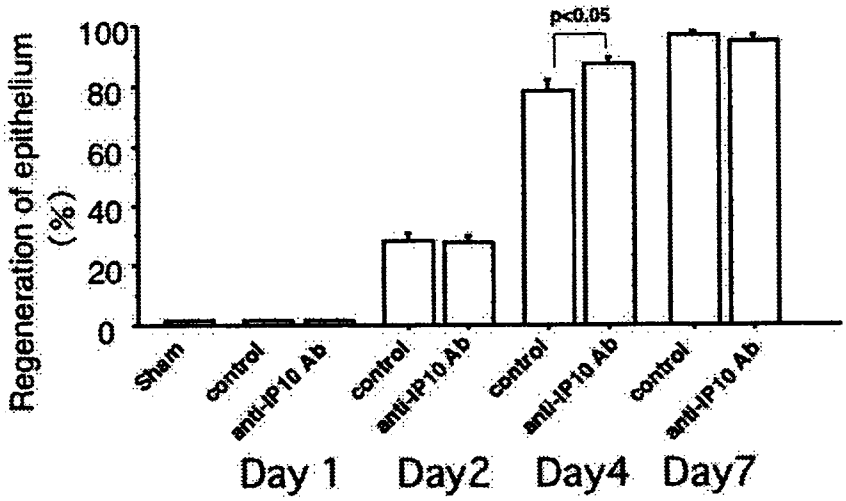



図 1　Values are the mean ± SEM.

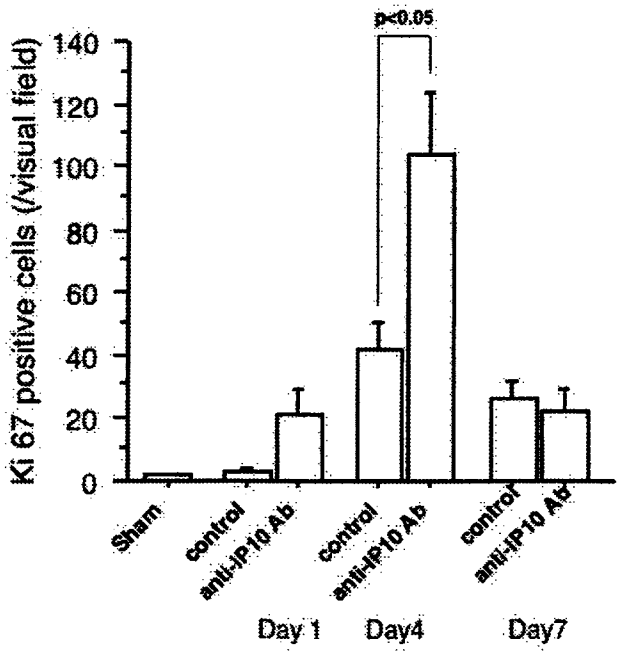



図２　Values are the mean ± SEM.

この IP-10/CXCR3 の腎障害時の役割をさらに検討する目的で，培養マウス尿細管上皮細胞を用いて wound healing test を施行した．その結果，抗 IP-10 中和抗体投与により wound healing が促進することが示された．さらに，抗 IP-10 中和抗体共培養により Ki67 陽性細胞で示される細胞増殖が促進された（図３）．

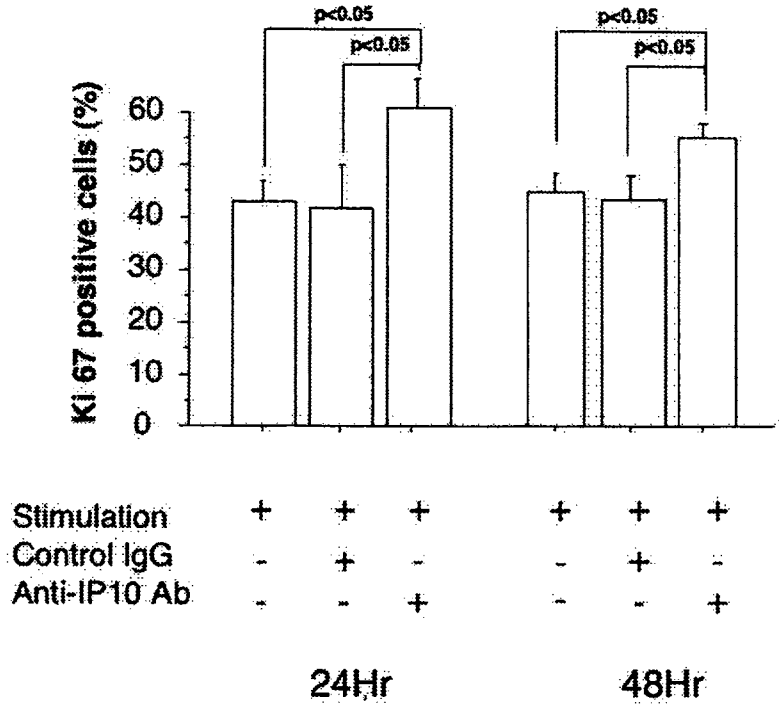



図 3　Values are the mean ± SEM.

これらの結果より，抗 IP-10 中和抗体投与により腎尿細管上皮細胞の再生ならびに増殖亢進が in vivo ならびに in vitro で確認された．以上より，IP-10/CXCR3 は腎発生に関与することに加えて，腎障害時に発現が亢進する IP-10/CXCR3 は腎尿細管上皮細胞の再生や増殖に関与することが推測された．

d) まとめ

IP-10/CXCR3 は腎の発生に関与するばかりではなく，腎障害時，とくに尿細管障害に修復/再生に深く関与する因子であることが推測される．

2) IP-10/CXCR3 の進行性腎線維化過程における意義

抄録

Backgroud. Fibrosis is a hallmark of progressive organ diseases. Interferon (IFN)-inducible protein of 10 kD (IP-10/CXCL10) is a potent chemoattractant for

activated T lymphocytes, natural killer (NK) cells, and monocytes. IP-10 also has several additional biologic activities via its receptor, CXCR3, however, the involvement of IP-10 in the pathogenesis of renal diseases remains unclear.

Methods. IP-10 and its receptor, CXCR3 were up-regulated in the course of progressive renal fibrosis in a UUO model. These findings were confirmed by evidence that mRNA expression of IP-10 and CXCR3 was increased in response to inflammatory stimuli. The impacts of IP-10 on renal fibrosis were investigated in a unilateral ureteral obstruction (UUO) model in CXCR3 deficient mice and mice treated with anti-IP-10 neutralizing monoclonal antibody (mAb). Anti-IP-10 mAb (5 mg/kg/day) was injected intravenously once a day until the day of sacrifice on days 1, 4 and 7. The effects of IP-10 were further confirmed in cultured tubular epithelial cells.

Results. Blockade of IP-10/CXCR3 signaling promotes renal fibrosis 4 and 7 days after ureteral ligation, as evidenced by increase in interstitial fibrosis as well as in hydroxyproline contents, concomitant decrease in hepatocyte growth factor (HGF) expression and converse increase in transforming growth factor (TGF)-$\beta_1$ in diseased kidneys. IP-10 blockade affected neither macrophage nor T cell infiltration in diseased kidneys in this model.

Conclusions. These results suggest that IP-10 via CXCR3 signaling is responsible for balancing profibrotic TGF-$\beta_1$ and anti-fibrotic HGF, and thus may contribute to eventual anti-fibrotic processes in the interstitium of the kidney.

## Introduction

Fibrosis is characteristic in progressive organ diseases, leading to organ failure. It is noted that fibrosis in interstitium is the determinant of the prognosis of renal diseases. Accumulating data on a molecular basis suggest that monocyte chemoattractant protein (MCP)-1/macrophage chemotactic and activating factor/CCL2-transforming growth factor (TGF)-$\beta_1$ axis is be a common regulatory pathway of chronic renal inflammation, resulting in renal fibrosis [1-4]. In contrast, physiological and adaptive mechanisms to prevent or cope with progressive fibrosis have been implicated. For examples, hepatocyte growth factor (HGF) ameliorates the initiation and progression of chronic fibrosis by the inhibition of TGF-$\beta_1$ expression in various experimental models [5-7], suggesting that delicate balance between TGF-$\beta_1$ and HGF activity in diseased kidneys may contribute to either fibrotic or antifibrotic events. Even though HGF is supposed to be a strong candidate for preventive mechanisms in renal fibrosis [6], molecular understandings involved in antifibrotic processes remain limited.

Interferon (IFN) -inducible protein of 10 kD (IP-10/CXCL10) identified as a product of genes induced in response to IFN-$\gamma$ is a well-known member of the CXC chemokine family [8]. Originally, IP-10 is described to be a potent chemoattractant for activated T lymphocytes, natural killer (NK) cells, and monocytes, participating in Th1 predominant immune response [9]. In addition to Th1 immune response, IP-10 via its cognate receptor, CXCR3 is also reported to be involved in human glomerulopathy, including mesangial proliferative glomerulonephritis (GN), rapidly progressive GN, membranoproliferative GN, lupus nephritis and nephrotoxic nephritis

[10-13]. Further, IP-10 plays a role in maintaining the podocyte function in glomeruli and anti-IP-10 antibody treatment exacerbates the glomerular alteration in Thy1.1 glomerulonephritis [14]. Therefore, a disturbed protective role of IP-10 from insults may contribute to alteration of glomerular lesions. In contrast, impacts of IP-10 on progressive interstitial lesions, including renal fibrosis, are poorly understood.

These findings prompted us to explore whether IP-10 takes part in protection from renal insults in progressive renal lesions. To address this issue, we examined the roles of IP-10 in renal fibrosis, characteristic to progressive renal lesions, in a unilateral ureteral obstruction (UUO) model in CXCR3 deficient mice and mice treated with anti-IP-10 neutralizing antibody. We describe here that blockade of IP-10/CXCR3 signaling promotes renal fibrosis, with concomitant decrease in HGF expression and reverse increase in TGF-$\beta_1$ in diseased kidneys.

## Materials and Methods

### Animals

Mice deficient in the expression of CXCR3 were generated by the process of gene targeting in murine embryonic stem cells and a breeding colony was maintained under specific pathogen-free condition [15]. Control male Balb/c mice, aged 8 weeks, were obtained from Charles River Japan (Atsugi, Kanagawa, Japan). All procedures used in the animal experiments complied with standards set out in the Guidelines for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Unilateral ureteral obstruction model

The general procedure of a UUO model is well described elsewhere [16]. In brief, CXCR3 deficient mice and wild-type mice were anesthetized with diethyl ether and pentobarbital sodium. A flank incision was made and the left ureter was ligated with 4-0 silk suture at two points. Sham operation was performed in a similar manner, except for left ureteral ligation. For pathological examination, both obstructed and contralateral kidneys were harvested from UUO animals 1, 4, and 7 days after ureteral ligation. CXCR3 deficient mice were sacrificed only 7 days after ureteral ligation. (N=6)

Blockade of IP-10 treated with anti-IP-10 antibody in renal fibrosis

To evaluate the impact of IP-10 on renal fibrosis as well as infiltrates in diseased kidneys, anti-IP-10 monoclonal antibody (mAb) [17] (5 mg/kg/day) or mouse IgG as a negative control was administered to Balb/c male mice intravenously 1 hour before ureteral ligation and injected once a day until the day of sacrifice. This antibody was obtained by immunizing mice with rat CXCL10/Fc fusion protein, and then screened by measuring the binding to the rat CXCL10/Ac2A fusion protein. In addition, it is confirmed that this mAb blocks mouse CXCL10-induced chemoattractive effect [18]. For pathological examination, obstructed kidneys as well as contralateral ones were harvested from UUO animals 1, 4 and 7 days after ureteral ligation (N=6, 8, 8 for each group at each time point). Sham-operated age-matched male Balb/c mice were used as a normal control (N=6).

Tissue preparation

One portion of the renal tissue was fixed in 10 % buffered formalin (pH 7.2), embedded in paraffin, cut at 4 μm, stained with hematoxylin and eosin, periodic acid Schiff's reagent (PAS), or Mallory-Azan and observed under a light microscope. Two independent observers with no prior knowledge of the experimental design evaluated each section. Mean interstitial fibrotic area, expressed as blue in Mallory-Azan staining, was evaluated from the whole area of cortex and outer medulla in the individual complete sagittal kidney section and expressed as percentage/$mm^2$ of the field by using Mac Scope version 6.02 (Mitani Shoji Co., Ltd., Fukui, Japan).

Immunohistochemical studies

The other portion of fresh renal tissue, embedded in O.C.T. compound and snap-frozen in n-hexane cooled with a mixture of dry ice and acetone, was cut at 6 μm on a cryostat (Tissue-Tek systems; Miles, Naperville, IL, USA). The presence of macrophages or T cells was detected immunohistochemically using rat anti-mouse F4/80 monoclonal antibody (clone A3-1; BMA Biomedicals AG, Augst, Switzerland) or rat anti-mouse CD3 monoclonal antibody (clone 17A2; R&D Systems, Minneapolis, MN, USA). Interstitial infiltrated F4/80-positive macrophages and CD3-positive T cells were counted in the whole area of the outer medulla, where cell migration was maximal, and expressed as the mean number ± standard error (SEM)/$mm^2$. The presence of IP-10 and CXCR3 was demonstrated immunohistochemically on

formalin-fixed, paraffin-embedded renal tissue specimens using the indirect avidin-biotinylated peroxidase complex method with rabbit anti-mouse IP-10 polyclonal antibodies (clone: sc-13951; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-mouse CXCR3 polyclonal antibodies (clone: sc-14641; Santa Cruz Biotechnology), respectively. Interstitial CXCR3-positive cells and IP-10-positive cells were also counted in the whole area of cortex and outer medulla, and expressed as the mean number ± standard error (SEM)/$mm^2$. The presence of TGF-$\beta_1$ was demonstrated immunohistochemically on formalin-fixed, paraffin-embedded renal tissue specimens using the indirect avidin-biotinylated peroxidase complex method with rabbit anti-mouse TGF-$\beta_1$ polyclonal antibodies (clone: sc-146; Santa Cruz Biotechnology). The positive area of TGF-$\beta_1$ was evaluated from the whole area of cortex and outer medulla, and expressed as percentage/$mm^2$ of the field by using Mac Scope version 6.02. To evaluate the specificity of these antibodies, tissue specimens were stained with normal rabbit IgG, or antibodies for TGF-$\beta_1$, IP-10, or CXCR3 absorbed with the excess amount of each molecule or a blocking peptide.

Dual staining

To determine the phenotypes of CXCR3-positive cells, dual-labeled immunohistochemistry was performed. In brief, formalin-fixed, paraffin-embedded renal tissue specimens were first incubated with rabbit anti-murine CXCR3 polyclonal antibodies (Santa Cruz), using the indirect avidin-biotinylated alkaline phosphatase complex method. After this process, specimens were incubated with rabbit

anti-murine TGF-$\beta_1$ polyclonal antibodies (Santa Cruz) using the indirect avidin-biotinylated peroxidase complex method.

Measurement of hydroxyproline contents in renal tissue

To further qualify renal fibrosis, hydroxyproline (HP) contents were measured according to a previous study [19]. Renal tissue was excised in 5-mm cubes and dried for 16 h at 120°C. HP amount was calculated by comparison to standards and the data were expressed as the amount per renal tissue (μg/mg tissue). Age-matched normal mice (n=6) were used as controls.

Proximal tubular epithelial cell culture and experimental procedure

Mouse proximal tubular epithelial cells (mProx24) [20] were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere (5% $CO_2$/95% air) at 37°C. Subconfluent cells were made quiescent by incubation with RPMI 1640 containing 0.1% FCS for 24 hours. Quiescent cells were incubated with 5 μM $H_2O_2$ in RPMI 1640 containing 0.1% FCS. For the induction of IP-10 and CXCR3, mProx24 cells were incubated with 5 μM $H_2O_2$ in the presence of INF-γ (100 ng/ml) and/or tumor necrosis factor (TNF)-α (25 ng/ml) for 24 and 72 hours.

Detection of transcripts of IP-10, the monokine induced by IFN-γ (Mig), IFN-inducible T cell α chemoattractant (I-TAC), CXCR3, TGF-$\beta_1$, and HGF in diseased kidneys and

cultured proximal tubular epithelial cells

To determine transcripts of IP-10, Mig, I-TAC, TGF-$\beta_1$ or HGF, total RNA was extracted from the whole kidney, in order to perform real-time reverse transcription polymerase chain reaction (RT-PCR). cDNA was reverse-transcripted from 1 μg total RNA from each mouse, by using a SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). Reverse transcription was performed using the following parameters: 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95 °C. Similarly, total mRNA extracted from cultured proximal tubular epithelial cells was analyzed for the detection of mRNA expression.

PCR amplifications are performed on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using 384-well microtiter plates. They are performed in a total volume of 20 μl, containing 1 μl cDNA sample, TaqMan Gene Expression Assays (Applied Biosystems) and TaqMan Universal PCR Mater Mix (Applied Biosystems), using the universal temperature cycles: 10 min at 94°C, following by 40 two temperature cycles (15 s at 94°C and 1 min at 60°C). Assay IDs of TaqMan Gene Expression Assays were Mm00438259_m1 for IP-10 [21], Mm00434946_m1 for Mig [22], Mm00444662_m1 for I-TAC [23], Mm00441724_m1 for TGF-$\beta_1$ [24], Mm001135185_m1 for HGF [25], and Mm00446953_m1 for beta-glucuronidase (GUS) [26]. mRNA expression of IP-10, TGF-$\beta_1$, and HGF in each sample was finally described after correction with GUS expression. No PCR product was detected in the real-time RT-PCR procedure without reverse transcription, indicating that the contamination of genomic DNA was negligible.

Gels of the PCR products after quantification of IP-10, TGF-$\beta_1$, HGF or GUS by real-time RT-PCR showed a single band (data not shown).

Similarly, to determine CXCR3 transcripts, the cDNA products from total RNA were amplified by PCR. Primers for CXCR3 (5'-GAACGTCAAGTGCTAGATGCCTCG-3' [sense]; 5'-GTACACGCAGAGCAGTGCG-3' [antisense]) [27] were used to detect CXCR3 transcripts. The housekeeping gene GAPDH was used for PCR controls. Scanner analysis of photographs of the DNA-stained agarose gels was evaluated by the band intensity comparison of GAPDH expression versus CXCR3 expression in computer image analysis.

Statistical analysis

The mean and SEM were calculated on all the parameters determined in this study. Statistical analyses were performed using Mann Whitney U test or Kruskal-Wallis test. $p<0.05$ was accepted as statistically significant.

Results

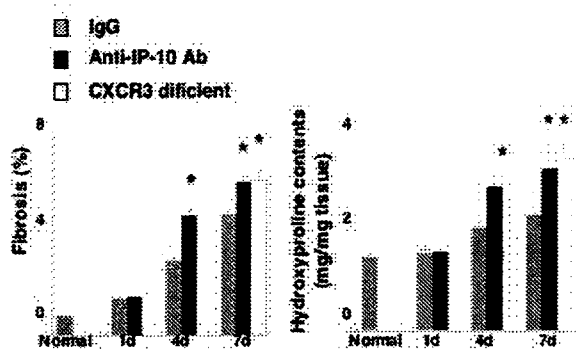
IP-10/CXCR3 expression in diseased kidneys in a UUO model

To determine the involvement of IP-10/CXCR3 signaling in real fibrosis, the presence of IP-10 and CXCR3 was evaluated immunohistochemically on days 1, 4 and 7 after unilateral ligation of the kidney. IP-10 was faintly detected in a normal mouse

kidney or a sham-operated mouse kidney (data not shown). In contrast, IP-10 expression was up-regulated especially in infiltrates in interstitium as well as tubular epithelial cells. Supporting this notion, IP-10 mRNA was increased, which reached a peak on day 4. Although up-regulated expression of IP-10 in diseased kidneys was not altered by the neutralization of IP-10 by the treatment of anti-IP-10 antibody。 On the other side, CXCR3, a cognate receptor for IP-10, was hardly detected in a normal mouse kidney either by an immunohistochemical analysis or by RT-PCR (Fig 2a, c, d). In contrast, CXCR3 was increased by ureteral ligation as evidenced by the increase in number of CXCR3-positive cells (Fig 2b, c) and by the enhanced expression of CXCR3 mRNA. IP-10 inhibition further increased the number of CXCR3-positive cells in interstitium on day 7 and the expression of CXCR3 mRNA on day 4.

IP-10 blockade exacerbated renal interstitial fibrosis

To determine the impact of IP-10 on progressive renal fibrosis, renal lesions expressed as blue in Mallory-Azan staining and HP contents were examined.




Values are the mean ± SEM. * $P<0.05$ and ** $P<0.01$ as compared with IgG-treated mice.

Normal mice or sham-operated mice hardly exhibited renal damage including renal fibrosis. Severe interstitial fibrosis was observed in the outer medulla in diseased kidneys in IgG-treated mice 4 and 7 days after ureteral ligation, as evidenced by increase in interstitial fibrosis and in HP contents. IP-10 inhibition further increased renal fibrosis in diseased kidneys induced by ureteral ligation as compared with that of mice without inhibition of IP-10 on days 4 and 7. Thus, IP-10 blockade promoted progressive renal interstitial fibrosis in kidney.

Increase in renal interstitial fibrosis in CXCR3 deficient mice

To confirm the impact of IP-10/CXCR3 signaling on progressive renal fibrosis, renal lesions were examined in CXCR3 deficient mice 7 days after ureteral ligation. Renal fibrosis was further increased in diseased kidneys in CXCR3 deficient mice, as evidenced by increase in interstitial fibrotic area as well as HP contents. It was similar to that observed in mice treated with anti-IP-10 mAb. Thus, IP-10/CXCR3 signaling appears to play a role in the pathogenesis of renal interstitial fibrosis.

Renal TGF-$\beta_1$ expression was increased by anti-IP-10 mAb treatment

To clarify the molecular mechanisms involved in increased fibrogenesis by IP-10 blockade, TGF-$\beta_1$, a potent fibrogenic molecule, was examined. Up-regulated TGF-$\beta_1$ protein was detected mainly in renal tubular epithelial cells and infiltrates in mice after ureteral ligation, as compared with that in normal mice or in sham-operated mice. Importantly, IP-10 inhibition up-regulated TGF-$\beta_1$ immunoreactivity in diseased kidneys on day 4. Similarly, TGF-$\beta_1$ mRNA expression was enhanced by ureteral ligation, which was further augmented by IP-10 inhibition on day 1. Furthermore, to determine the TGF-$\beta_1$ expression in CXCR3- positive cells, dual staining was performed. Dual positive cells for CXCR3 and TGF-$\beta_1$ were detected in renal tubular cells and infiltrates by ureteral ligation, which was further augmented by IP-10 inhibition on days 4 and 7. Thus, the blockade of IP-10 up-regulated the expression of TGF- $\beta_1$, which might, in turn, contribute to the increase in fibrogenesis in diseased kidneys.

Anti-IP-10 mAb treatment reduced transcripts of HGF in diseased kidneys

Transcripts of HGF, a potent antifibrogenic factor, were further evaluated in diseased kidneys. Transcripts of HGF were up-regulated in diseased kidneys by real-time RT-PCR, whereas these were faintly detected in a normal mouse kidney or a sham-operated mouse kidney. In contrast, transcripts of HGF in diseased kidneys were less induced by IP-10 blockade on day 4. Thus, the blockade of IP-10 inhibited the expression of HGF, which might result in the increase in fibrogenesis in kidney.

Interstitial F4/80-positive macrophages and CD3-positive T lymphocytes were not affected by anti-IP-10 mAb treatment

Since IP-10 is a potent chemoattractant for activated T lymphocytes and macrophages, whether IP-10 has impacts on interstitial cell infiltration was examined. Positive cells for F4/80 or CD3 were counted on days 1, 4 and 7. F4/80-positive macrophages infiltrated mainly in the outer medulla in diseased kidneys. The number of infiltrated F4/80-positive macrophages in interstitium did not differ by anti-IP-10 mAb treatment at any time point after ureteral ligation. Similar to F4/80-positive macrophages, the number of infiltrated CD3-positive T lymphocytes in diseased kidneys in mice treated with anti-IP-10 mAb was not statistically different from that observed in mice treated with IgG. Therefore, IP-10 blockade affected neither macrophage nor T cell infiltration in diseased kidneys at least in this particular model.

Anti-IP-10 mAb treatment reduced transcripts of Mig in diseased kidneys

To determine whether Mig and I-TAC, other ligands of CXCR3, were concerned with anti-IP-10 treatment, transcripts of Mig and I-TAC were evaluated by real-time RT PCR. The expressions of Mig and I-TAC were hardly detected in a normal mouse kidney by real-time RT-PCR. In contrast, the expression of Mig and I-TAC was increased by ureteral ligation. Transcripts of Mig in diseased kidneys were down-regulated by IP-10 blockade on day 4, whereas IP-10 inhibition failed to change the expression of I-TAC.

Expression of IP-10/CXCR3 in cultured renal tubular epithelial cells

Next, whether tubular epithelial cells are capable of producing IP-10 or expressing CXCR3 in response to inflammatory stimuli including INF-γ, TNF-α and $H_2O_2$ was examined in mProx24 cells. Transcripts of IP-10 were faintly detected in mProx24 cells without stimulation. However, transcripts of IP-10 were up-regulated in response to INF-γ, which was further augmentated by the co-existence of TNF-α. Similarly, CXCR3 expression was hardly detected without stimulation. By contrast, INF-γ enhanced the expression of CXCR3 mRNA. These results are confirmative to our findings that IP-10 and CXCR3 were up-regulated in tubular epithelial cells in diseased kidneys in a UUO model.

Discussion

In the present study, we explored to determine the inhibitory impacts of IP-10 by

anti-IP-10 mAb treatment on renal fibrosis induced by ureteral ligation. We now report that inhibition of IP-10 promoted progressive renal fibrosis, concomitantly with up-regulation of TGF-$\beta_1$ and decrease in HGF expression in diseased kidneys. Increase in renal fibrosis was also confirmed in CXCR3 deficient mice. We also noted that anti-IP-10 mAb treatment affected neither F4/80-positive macrophages nor CD3-positive T cell, which infiltrated into the diseased kidneys. Taken together, we presume that IP-10 is required for anti-fibrotic process involved in progressive renal insults via balancing HGF and TGF-$\beta_1$.

The most compelling part of this study is that blockade of IP-10 activity promoted renal fibrosis. Renal fibrosis, the characteristic to progressive renal disease, is the determinant of prognosis of renal diseases. Therefore, it is of importance to clarify molecular mechanisms involved in renal fibrosis, thereby establishing the therapeutic strategies for renal fibrosis. Thus far, MCP-1/TGF-$\beta_1$ axis has been established to a key role in fibrogenic responses in kidney [1,2]. Supporting this notion, an intrinsic regulatory loop in which MCP-1 stimulates TGF-$\beta_1$ production by resident glomerular cells has been suggested in the absence of infiltrating immune competent cells [28]. Further, MCP-1 blockade reduced renal fibrosis, with the concomitant decrease in TGF-$\beta_1$ expression [3,4,29]. In the present study, IP-10 blockade up-regulated TGF-$\beta_1$ expression in diseased kidneys. IP-10 production was strongly induced in the presence of IFN-$\gamma$ and TNF-$\alpha$, both of which are known to be key molecules in renal fibrosis [30-32]. Collectively, once renal insults including IFN-$\gamma$ and/or TNF-$\alpha$ activate tubular epithelial cells, IP-10 expression is up-regulated.

Thus, IP-10 might have a protective role for renal fibrosis to quench TGF-$\beta_1$-associated fibrotic processes.

To examine whether IP-10 blockade has an impact on HGF mRNA expression was analyzed in diseased kidneys. HGF has been reported to attenuate renal fibrosis via the suppression of TGF- and platelet-derived growth factor [30-32]. We have uncovered down-regulation of HGF expression in diseased kidneys by IP-10 blockade, possibly resulting in promoting renal fibrosis. Whether TGF- $_1$ directly reduces HGF expression in renal tubular epithelial cells remains unclear at present. In addition, it is not clear whether IP-10 induces HGF expression in tubular epithelial cells. In this study, HGF expression was not detected in tubular epithelial cells by real-time RT PCR. However, IP-10 expression in response to IFN- and/or TNF- might contribute to antifibrotic process, with the up-regulation of HGF in addition to the decrease in TGF- in diseased kidneys.

In this manuscript, anti-IP-10 mAb treatment did not affect the infiltration of immune competent cells, including T cells and macrophages on which CXCR3 is expressed. Consistent with these, the blockade of IP-10 hardly affected mRNA expression of IP-10, Mig and I-TAC. Recent studies revealed that neutralization of IP-10 decreases the infiltration of T cells in diseased organs, including kidneys [15,33,34]. Therefore, the dose of mAb examined in this study might not be sufficient to prevent the infiltration of T cells or macrophages. However our findings are consistent with the previous report that blockade of IP-10 enhanced the urinary levels of protein of Thy 1.1 glomerulonephritis [14]. In this report, there were no differences

in numbers of ED1- and CD5-positive inflammatory cells in glomeruli by anti-IP-10 mAb treatment, suggesting that IP-10 contributes to maintaining the structure and the function of podocytes, not to inflammatory cells. In this study, CXCR3 was also up-regulated in response to IFN- in cultured tubular epithelial cells as well as in diseased kidneys. Therefore, anti-IP-10 mAb treatment may have an impact on CXCR3, at least, expressed on tubular epithelial cells, and not on immune competent cells. Taken together, progressive renal fibrosis caused by IP-10 blockade might not result from the modulated inflammatory responses.

In conclusion, blockade of IP-10/CXCR3 promoted renal fibrosis possibly by disturbing the antifibrotic mechanism mediated by in TGF- and HGF. Thus, IP-10/CXCR3 might contribute to an antifibrotic therapeutic strategy in progressive renal fibrosis.

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3) 総括

IP-10/CXCR3 は腎発生に関与するばかりではなく,腎線維化に至る修復機構に深く関与する因子であることが推測される. さらに腎における抗線維化療法の新

しい標的分子としてIP-10/CXCR3の臨床応用が期待される.