筆者らの検討により，動物実験のレベルでその重要性が示されてきた制御性T細胞・PD-1が，臨床の免疫寛容にも重要である可能性が示唆された．一方，γδT細胞は，臨床から得られた事実により免疫寛容との関連性が示唆された．1施設で100名近い免疫寛容の患者がフォーローアップされているのは世界でも京都大学だけである．それゆえに，筆者らは臨床の免疫寛容のメカニズム解明へ向けて全力を尽くさなければならない．

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小柴貴明：1967年生まれ．'92年，京都大学医学部卒業．'98年から，ベルギー・ルーベンに留学して，ラット小腸移植・心臓移植モデルを用いて，制御性T細胞による免疫寛容，制御性T細胞と慢性拒絶，免疫抑制剤の免疫寛容に及ぼす影響について研究した．2002年帰国後，生体肝移植後の免疫寛容のメカニズムの解明と大動物を用いた免疫寛容の誘導，免疫寛容を誘導する次世代の薬剤の開発をテーマに研究を進めている．

X．臓器移植免疫寛容における制御性T細胞

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肝移植では他臓器と違い，臓器移植の最終目標である免疫寛容が自然に起こりうる（immuno-privilege）。特に京都大学の小児生体肝移植では免疫寛容の成立が高い頻度で起きたため，そのメカニズムを調べる機会に恵まれた。免疫寛容患者の末梢血でドナーの抗原に特異的な免疫制御を果たす制御性T細胞（Tregs）が増加しており，また移植肝にもTregsが存在した。局所でTregsが移植肝を拒絶から守っている可能性がある。Tregsが肝のimmuno-privilegeという現象を説明できるのか，今後の検討を要す。

制御性T細胞／免疫寛容／肝移植

Ⅰ．臓器移植における免疫寛容の必要性・課題

各種臓器移植において拒絶反応を防ぐために免疫抑制剤が投与される。しかし，免疫抑制剤による非特異的な免疫の抑制下に患者は感染症に罹患する危険にさらされるだけではなく，免疫抑制剤そのものの副作用も大きな弊害である。従って，免疫抑制剤を投与されなくても免疫が制御され拒絶が起こらない状態，すなわち免疫寛容が成立することは臓器を問わず患者にとって大変に好ましい[1)]。

とりわけ小腸移植と肺移植の移植後の5年生存率は50%前後と他の臓器の移植より低く，**図1**に示すように肺癌を除く主要な悪性腫瘍の予後よりも悪い。従って，これらの臓器の移植の予後を改善することは，緊急の課題である。小腸移植と肺移植では拒絶反応が強く起こるため，これまで免疫抑制剤が強力に使用されてきた。しかし，その結果，免疫抑制剤の弊害が強く現れ，患者が死亡することが多い。従って，今後，特にこのような臓器で免疫寛容が誘導されれば患者の生存率は著しく向上すると期待される。

また，**図1**が示すように肝移植の場合，全体的な予後は良好であるが，原疾患により予後が異なる。欧米，日本どちらにおいても現在，成人の肝移植の適応の1番はC型肝硬変である。ガンマグロブリンなど特効薬のないC型肝炎ウイルスは移

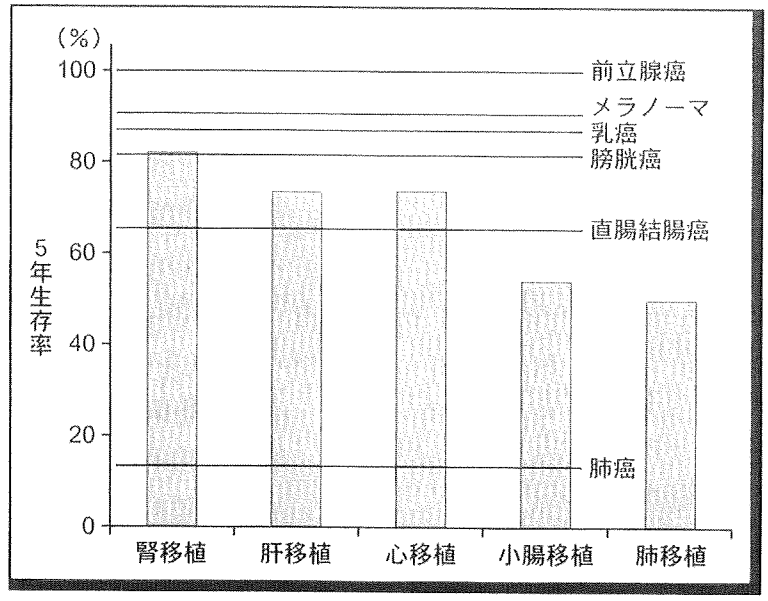

図1　5年生存率 臓器移植 対 悪性腫瘍

腎・肝・心移植の予後は主要な悪性腫瘍の予後と同等である．しかし，小腸・肺移植の予後は不良である．

(2005 OPTN/SRTR Annual Report〔Organ Procurement and Transplant Network〕Cancer statistics 2006〔American Cancer Society〕)

植を行っても，血液中に残存したウイルスが移植肝に再感染を起こすことは必発である。さらに移植後の免疫抑制剤の使用により，ウイルスの増殖は促進され，移植肝のウイルスによる変化が急速に進行すると考えられている[2]。事実，移植後，短期間で移植肝が肝硬変となることはまれではない。従って，C型肝硬変で移植を受けた患者で仮に免疫寛容が成立すれば移植肝のウイルスによる変化を遅らせることができるのではないかと予想される。

Ⅱ．肝移植における免疫寛容

マウス，ラット，ブタを用いた動物実験では，ドナーとレシピエントの主要組織適合性抗原の違いを越えて移植肝が免疫抑制剤を使用しなくても拒絶されない場合がある[3]~[5]。ところが，同じ系のドナーとレシピエントの組み合わせで腎臓や心臓の移植をすれば拒絶が起こる。この現象は肝臓の immuno-privilege と呼ばれる。同様に，ヒトの腎臓移植や心臓移植で免疫寛容が成立することは例外的である。しかし，京都大学で生体肝移植を受けた小児の患者において，免疫抑制剤による EB (Epstein-Barr) ウイルスの再燃や，免疫抑制剤の副作用が原因で，免疫抑制剤を中止したところ，拒絶が起きず免疫寛容の成立した患者が見られた。これらの経験から，京都大学では小児の患者に限って肝機能の安定している場合には，計画的に免疫抑制剤を中止してきた[6]。これまで80例以上の患者に免疫寛容が成立した（小児の肝移植患者は総計およそ600例）[7]。しかし，この

EB (Epstein-Barr)

immuno-privilegeという現象を十分に説明する機序は明らかにされていない。

Ⅲ．免疫寛容のメカニズム解明

免疫寛容のメカニズム解明をすることには，2つの理由が存在する。1つは，免疫抑制剤を減量する過程で拒絶が起きる可能性があり，拒絶の危険を回避し免疫抑制剤を中止する指標を得るために，メカニズムが解析されなければならない。2つ目は，メカニズムが詳細に解明されれば，移植患者に積極的に免疫寛容を誘導する新たな方法を開発するための手がかりになる。著者らは，制御性T細胞が生体肝移植後の免疫寛容に重要な役割を果たしているのか，もし，そうであればどのようにして，拒絶を抑制しているのかを調べてきた。

Ⅳ．免疫寛容患者における制御性T細胞の役割

免疫抑制剤を完全に中止して，1年以上肝機能が安定している患者について末梢血，移植肝内における制御性T細胞の存在と機能について詳細な検討を行った。

1．末梢血における$CD4^+CD25^{high+}$細胞

免疫寛容患者の末梢血におけるリンパ球中の$CD4^+CD25^{high+}$細胞の割合をフローサイトメータの手法を用いて解析した。長年，Suppressor cellsという概念が存在はしたが，その存在をフェノタイプで同定することが出来なかった。しかし，共著者の坂口により，マウスの免疫抑制性の細胞集団を$CD4^+CD25^{high+}$細胞というフェノタイプで同定し，今日，$CD4^+CD25^{high+}$細胞は制御性T細胞（regulatory T cells；Tregs）と呼

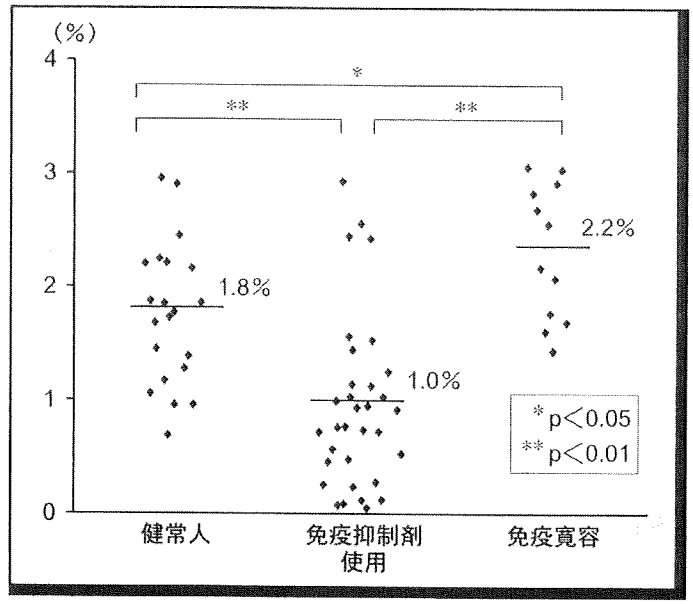

図2　免疫寛容患者における$CD4^+CD25^{high+}$細胞の増加

Tregs（$CD4^+CD25^{high+}$細胞）の割合は生体肝移植後の免疫寛容の患者の末梢血で増加していた．

（文献8より引用）

ばれるようになった。SPFにいるマウスではCD4$^+$CD25$^{intermediate+}$細胞（エフェクター細胞）は事実上存在しないので，CD4$^+$CD25$^-$細胞とCD4$^+$CD25^{high+}細胞の区別は難しくはない。しかし，pathogensに曝露を受けエフェクター細胞の多いヒトでは，CD4$^+$CD25$^{intermediate+}$細胞が存在して，CD4$^+$CD25^{high+}細胞の同定は難しい。著者らはpathogensに曝露を受けず，CD4$^+$CD25$^{intermediate+}$細胞の存在しない新生児の血液を臍帯から採取して，CD4$^+$CD25^{high+}細胞のCD25のintensityを定義した。そうして，定義されたリンパ球中のCD4$^+$CD25^{high+}細胞の割合は，免疫寛容の患者において，免疫抑制剤使用中の患者や同年代の健常人に比較して有意に増加していた（**図2**）[81]。

2．末梢血CD4$^+$CD25^{high+}細胞の免疫制御機能

さらに，細胞分離装置にて分離した免疫寛容患者のCD4$^+$CD25$^-$細胞とCD4$^+$CD25^{high+}細胞を**図3**に示すように混合する割合を変えて，ドナーの抗原と3rd partyの抗原に対する，MLR（mixed lymphocyte reaction）を測定した。3rd partyの抗原に対するCD4$^+$CD25$^-$細胞のMLRはCD4$^+$CD25$^-$細胞：CD4$^+$CD25^{high+}細胞を1：1から1：1/3まで，CD4$^+$CD25^{high+}細胞により抑制されている。ところが，ドナーの抗原に対するCD4$^+$CD25$^-$細胞のMLRはCD4$^+$CD25$^-$細胞：CD4$^+$CD25^{high+}細胞を1：1から1：1/9まで，CD4$^+$CD25^{high+}細胞により抑制されており，1：1/27になって初めて解除される（**図3**）。このことは，免疫寛容患者のCD4$^+$CD25^{high+}細胞がCD4$^+$CD25$^-$細胞のMLRを，ドナー抗原に対して，3rd partyの抗原に対するよりも強く抑制していることを意味する。換言すれば，免疫寛容患者のCD4$^+$CD25^{high+}細胞の免疫制御はドナー抗原特異的である。本検討では，5名の免疫寛容患者のうち4名に，ドナー抗原特

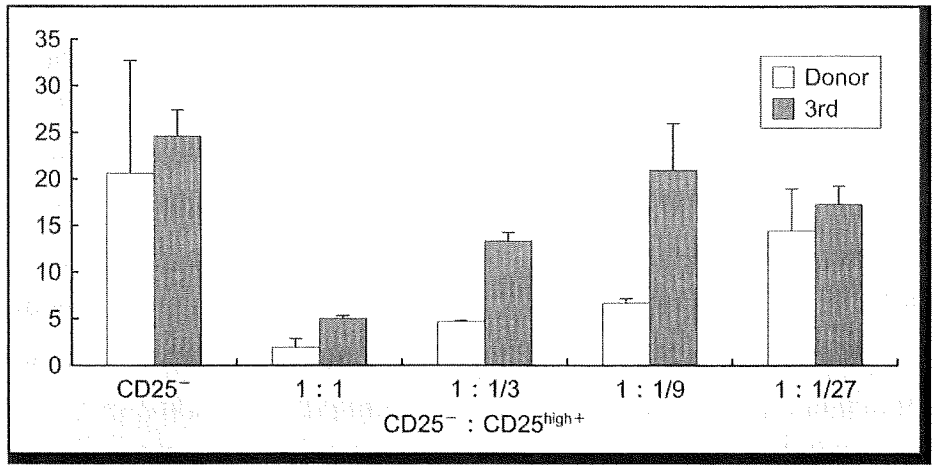

図3　免疫寛容患者におけるCD4$^+$CD25^{high+}細胞によるドナー抗原に特異的な免疫制御

3rd partyに対するMLRはCD25$^-$：CD25^{high+}が1：1/9で抑制が解除される．一方ドナーに対しては1：1/27で抑制が解除される．

（文献7より引用）

Tregs（regulatory T cells；制御性T細胞）　　MLR（mixed lymphocyte reaction）

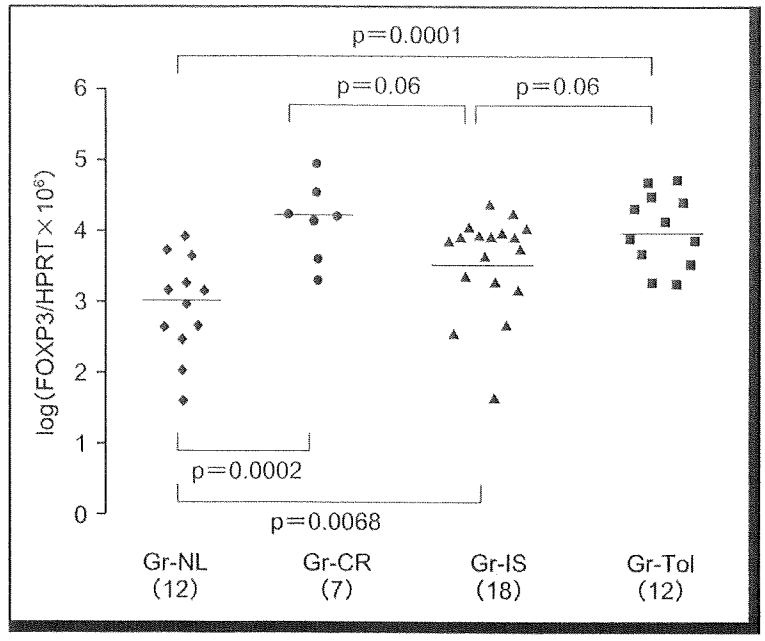

図4　移植肝内の *FOXP3* mRNA の発現

FOXP3 mRNA の発現は Gr-Tol と Gr-CR の両方で増加していた．Gr-NL：正常肝，Gr-CR：慢性拒絶，Gr-IS：免疫抑制剤使用中（減量前），Gr-Tol：免疫寛容．—は各群の mean value を示す．

（文献 11 より引用）

異的制御性T細胞の存在が証明された[7]。

3．移植肝内の FOXP3⁺細胞

京都大学では，2003 年から肝機能が正常でも長期生存症例にプロトコール生検で移植肝の病理学的な検討を行っている。免疫寛容の患者のプロトコール生検では，移植肝に急性，慢性拒絶を認めなかった[9]。同時に，著者らは，免疫寛容の患者のプロトコール生検により，移植肝内の制御性T細胞の存在について検討を行う機会を与えられた。FOXP3 は制御性T細胞の発生と分化に不可欠な転写因子であり，今日存在する最も信頼できる制御性T細胞のバイオマーカーである。著者らはまず，生検組織から，Total RNA を抽出し，mRNA レベルで FOXP3 の発現を検討した。その結果，**図4**に示すように *FOXP3* mRNA の発現は免疫寛容の患者で，免疫抑制剤内服中（減量前）の患者に比べ増加していた。また，*FOXP3* は mRNA レベルでは，慢性拒絶で喪失した移植肝でも，免疫寛容の移植肝と同程度に増加していた（**図4**）。さらに，免疫染色を行い CD4⁺，CD8⁺，FOXP3⁺（蛋白レベル）細胞の分布と，数について検討を行った。免疫寛容の患者の移植肝，免疫抑制剤内服中（減量前）の患者の移植肝の門脈域に CD4⁺と CD8⁺の集ぞくが散見された。門脈域における CD4⁺と CD8⁺の密度は，免疫寛容の患者と免疫抑制剤内服中の患者で差がなかったが，FOXP3⁺の密度は，免疫寛容の患者で，免疫抑制剤内服中の患者に比べて有意に増加していた（**図5**）。慢性拒絶の移植肝には門脈域に CD8⁺細胞が豊富に見られた。しかし，*FOXP3* mRNA の発現とは違い，FOXP3⁺は発現を認めなかった（**図5**）。また，CD4，FOXP3，および CD8，FOXP3 の二重蛍光染色を行ったところ，FOXP3⁺細胞は 92%が CD4⁺で CD8⁺はわずかに 8%であった（**図6**）。

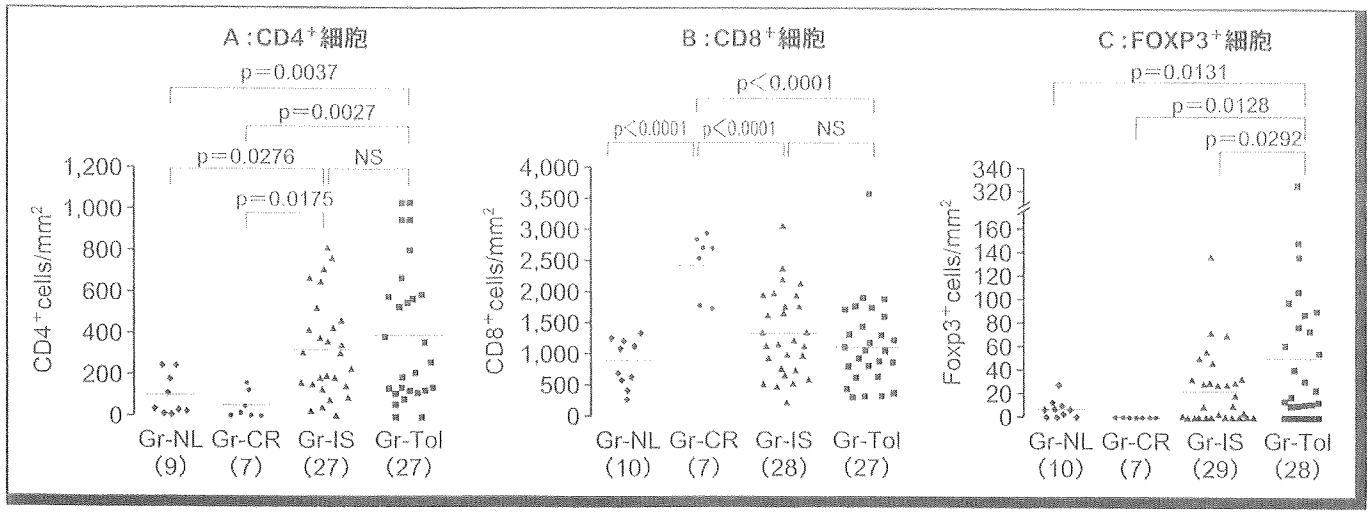

図5　移植肝内のCD4⁺，CD8⁺，FOXP3⁺細胞の数

CD4⁺，CD8⁺の数はGr-IS，Gr-Tolの間で差がなかったが，FOXP3⁺の数はGr-Tolで増加していた．

Gr-NL：正常肝，Gr-CR：慢性拒絶，Gr-IS：免疫抑制剤使用中（減量前），Gr-Tol：免疫寛容．—は各群のmean valueを示す．

（文献9，11より引用）

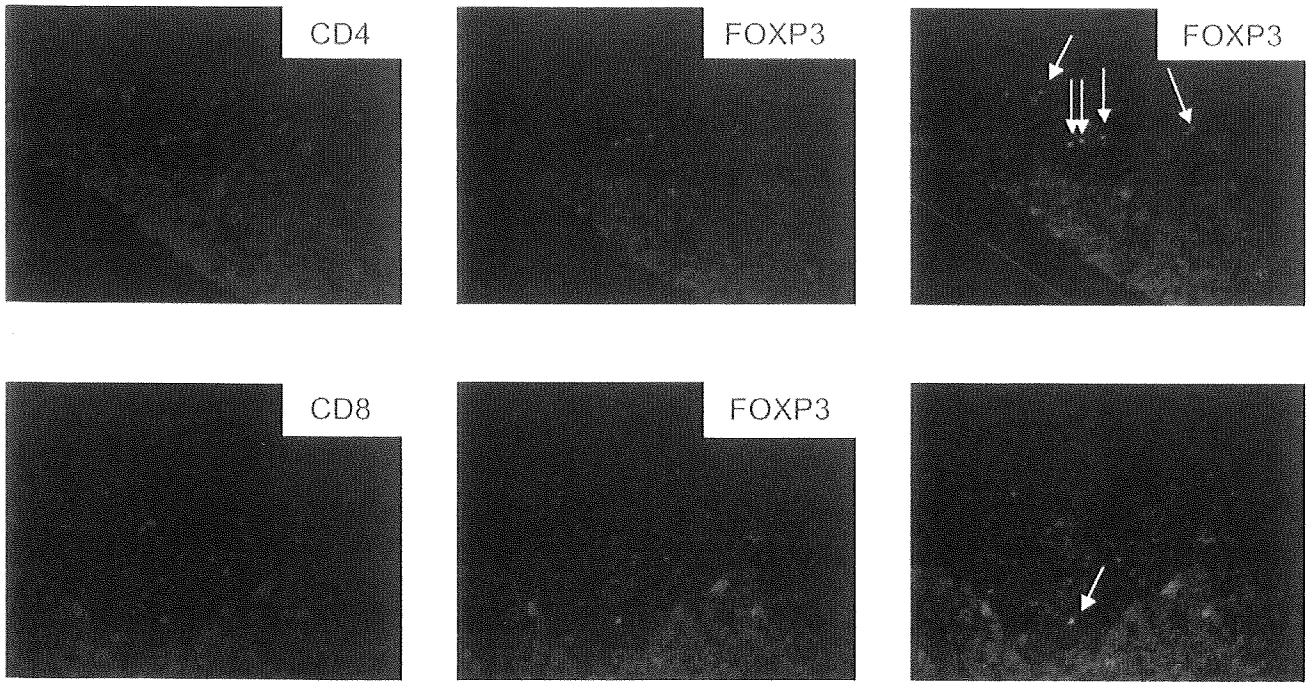

図6　CD4，FOXP3及びCD8，FOXP3の二重蛍光染色

左上；赤CD4⁺細胞，中央上；緑FOXP3⁺細胞，右上；矢印の黄緑CD4⁺FOXP3⁺細胞

左下；赤CD8⁺細胞，中央下；緑FOXP3⁺細胞，右下；矢印の黄緑CD8⁺FOXP3⁺細胞

免疫寛容患者の移植肝内に存在するFOXP3⁺細胞のうち，92%はCD4⁺，8%はCD8⁺であった．

（文献11より引用）

すでに，免疫寛容患者の末梢血の中でのリンパ球中の $CD4^+CD25^{high+}$ 細胞の割合の増加と抗原特異的制御性T細胞の存在が同定されていることを合わせて考えると，免疫寛容の患者の移植肝には $CD4^+FOXP3^+$ 制御性T細胞が存在して，拒絶から移植肝を守っているのではないかとの仮説が成り立つ。一方，免疫寛容のバイオマーカーという見地から *FOXP3* mRNA，とFOXP3（蛋白）を捉えた場合，*FOXP3* mRNAは拒絶の移植肝にも高発現しているが，FOXP3（蛋白）が増加しているのは免疫寛容だけなので，著者らはFOXP3（蛋白）の方が，免疫寛容のバイオマーカーの候補となりうると考えている。しかし，**図6**に示すように，免疫寛容の移植肝にもFOXP3（蛋白）が認めない症例があり，このマーカーが免疫寛容のバイオマーカーとして，有用かどうかということについてはプロスペクティヴは検討を要する。

ロッテルダムのグループは心臓移植の拒絶時，生検組織内の *FOXP3* mRNAが高発現することを報告している[10]。著者らが認めた慢性拒絶の移植肝内での *FOXP3* mRNAの高発現に一致する所見である。それにも関わらず，$FOXP3^+$（蛋白）細胞は，慢性拒絶の肝臓では認めなかった。最近，ヒトのエフェクター細胞（$CD4^+CD25^-$細胞，$CD8^+CD25^-$細胞）は活性化するとlow levelの *FOXP3* mRNAを発現することが *in vitro* の研究で明らかになった。すでに，慢性拒絶の移植肝には $CD8^+$ 細胞が豊富に存在することが分かっている。これらの細胞は拒絶時に活性化されていると考えられ，low levelの *FOXP3* mRNAを発現する可能性がある。仮に，個々の細胞の発現levelが低くても，細胞数が多ければ，慢性拒絶の移植肝内の *FOXP3* mRNAのトータルは増加しているであろう。しかし，免疫染色では，その感度の低さからlow levelのFOXP3（蛋白）は検出されない。このように考えると，慢性拒絶の移植肝においてmRNAのレベルで検出されたFOXP3遺伝子のコードする蛋白が，免疫染色のレベルでは検出されなかった理由を説明することが出来る[11]。

ま と め

京都大学で生体肝移植を受け，免疫寛容の成立した患者の末梢血では，ドナー抗原特異的免疫制御能を有する制御性T細胞が増加しており，移植肝でも制御性T細胞が増加していた。これらのデータから，制御性T細胞は移植肝の局所で拒絶から移植肝を守ることで免疫寛容が維持されている可能性が示唆された。肝臓のimmuno-privilegeと制御性T細胞との関係を明らかにするため更なる研究を要する。

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Requirement of Protocol Biopsy Before and After Complete Cessation of Immunosuppression After Liver Transplantation

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Background. Operational tolerance is defined as long-term acceptance of a transplanted organ after complete cessation of immunosuppression (IS), but may not always protect against antigen-dependent changes in graft morphology.
Method. IS free patients after living-donor liver transplantation (LDLT) underwent protocol biopsy (tolerance group [Gr-Tol]) and were evaluated for rejection and fibrosis. The degree of fibrosis was compared with those in the patients on maintenance IS group (Gr-IS) and the base line normal liver group (Gr-BS). When bridging fibrosis or progression of fibrosis was observed, IS was reintroduced or increased in Gr-Tol or in the patients in the weaning process.
Results. Neither acute nor chronic rejection was observed. The degree of fibrosis, however, was significantly greater in Gr-Tol than those in Gr-IS and Gr-BS. In Gr-Tol, the number of graft infiltrating $FOXP3^+$ cells was significantly increased, the interval between LDLT and biopsy plus the donor age was significantly longer, and recipient age at LDLT was significantly younger, compared with those in Gr-IS. However, none of these three parameters correlated with the degree of fibrosis. In 7 of 11 patients in whom IS was reintroduced or increased, the improvement of fibrosis was observed by the subsequent biopsy.
Conclusion. Grafts of operationally tolerant patients after LDLT did not exhibit acute or chronic rejection, but they exhibited fibrosis. It remains elusive whether fibrosis observed in tolerant grafts is antigen dependent. The finding that the reintroduction or the increase of IS fibrosis was improved supported the possibility that fibrosis in operationally tolerant after patients was antigen dependent.

Keywords: Protocol biopsy, Tolerance, Liver transplantation.

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In Japan, living-donor liver transplantation (LDLT) has saved lives of many patients suffering from end-stage liver diseases, and improved the quality of their lives (*1*). However, a problem which has remained unsolved is the dependency of the patients on nonspecific life-long immunosuppression (IS) with its attending complications (*2*). Therefore, establishment of operational tolerance (defined as the long-term acceptance/function of a transplanted organ after complete cessation of IS) (*3, 4*) may lead to considerable progress (*5*). We have developed an elective protocol that enables a substantial proportion (approximately 40%) of liver transplantation patients to be weaned from IS (*6*). In addition to those patients, some patients had to stop IS nonelectively because of severe complications of IS, but they had not exhibited any

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clinical sign of rejection for a long time (*6*). Collectively, we found that operational tolerance occurred in the significantly higher proportion (15% of all the pediatric patients), regardless of whether IS was weaned-off electively or nonelectively, in comparison with other transplant centers (*7, 8*). However, protocol biopsy has never been performed either before or after complete cessation of IS in those tolerant patients. After the aforementioned definition of operational tolerance, there are no strictly defined histologic criteria of tolerance (*3, 4*). Thus, a question arose as to whether establishment of operational tolerance, which offers protection against clinically manifest rejection (the presence of a normal liver test), similarly offers protection against the antigen-dependent histologic changes of grafts characterized by cellular infiltrates, vessels damages, fibrosis, and the changes of the bile ducts.

To address this question, protocol biopsy was performed on our operationally tolerant patients, and histology of accepted grafts was studied in detail. This is the first report providing detailed evidence that operationally tolerant patients, despite a normal or a quasi-normal liver test, exhibited some morphological changes in accepted grafts, which are distinguishable from those of patients on maintenance IS and the normal liver.

METHOD

Operational Tolerance

From June 15, 1990 to May 20, 2005, 659 cases of LDLT were performed on 581 pediatric patients (<18 years old) at the Kyoto University Hospital. Eighty-eight patients (15.0% of all the pediatric patients) were weaned from IS (=operational tolerance) (*7*). Fifty-five of the 88 tolerant recipients were weaned from IS by our elective protocol (*7*). The other 33 patients stopped IS nonelectively (30 patients because of severe complications of IS such as Epstein-Barr virus infection, and three patients due to noncompliance) (*7*). On the contrary, 22 patients failed to stop IS. In those patients, during the weaning process or after cessation of IS, serum transaminases were elevated and IS was reintroduced with or without confirmation of rejection by biopsy.

Immunosuppression Protocol

Patients were basically immunosuppressed with tacrolimus and low dose of steroids. Cyclosporine A was intravenously administered continuously with steroids for the first14 cases, and then it was switched to tacrolimus for all the subsequent patients because of hypertension and an increase in serum blood urea nitrogen level. In our current protocol, the target trough level of tacrolimus was 10 to 15 ng/mL for the first 2 weeks and 5 to 10 ng/mL for the following 2 months. After discharge, the dose of tacrolimus was determined individually depending on each patient's condition.

The initial steroid was methylprednisolone, which was intravenously administered at a dosage of 10 mg/kg immediately after reperfusion, followed by a dosage of 1 mg/kg twice daily for the first 3 postoperative days, 0.5 mg/kg twice daily for the next 3 days, and 0.3 mg/kg on day 7. From day 8, predonisolone was started to be given orally at a dosage of 0.3 mg/kg/day, which was gradually tapered and then was stopped in 3 months.

When patients fulfilled following criteria, they began the weaning protocol; (1) 2 years posttransplant, (2) a normal graft function, and (3) no single episode of rejection for 1 year. The frequency of tacrolimus administration was reduced as followings: conventional two times per day, once per day, four times per week, three times per week, twice per week, once a week, twice a month, once a month, and finally it was stopped. As long as a liver function remained normal, the interval of each step was 3 to 6 months (*6*).

Protocol Biopsy

Since January 2003, protocol biopsy has been performed on our pediatric patients 1, 2, 5, and 10 years after LDLT. The Ethical Committee of the Faculty of Medicine, the Kyoto University approved protocol biopsy which was performed in the presence of a normal liver test. Informed consent was obtained from the patients following the declaration of Helsinki. Parental permission was obtained in case of those patients were younger than 20 years old. The specimens of liver grafts were obtained by core needle biopsy (17 gauge) using ultrasonography under local anesthesia.

Histological Evaluation

The liver specimens were fixed in 10% buffered formalin, processed routinely, and sliced into 3-μm paraffin sections. The staining methods included hematoxylin and eosin, Masson trichrome, and cytokeratine 7 (OV-TL 12/30, Dako, Denmark, dilution 1:200). Acute rejection and chronic rejection were evaluated following the Banff Schema (*9*). On cytokeratine 7 stained sections, whether loss of the interlobular bile ducts existed or not was examined. The degree of liver fibrosis was quantified on Masson trichrome stained sections following the Ishak's modified staging (*10*). Each evaluation was blindly conducted by pathologists (H.S. and H.H.).

Immunohistochemistry and Double Immunofluorescent Staining

Four-micrometer paraffin embedded sections were deparaffinized and rehydrated before antigens were retrieved using retrieval solution (Dakocytomation, Glostrup, Denmark). For immunohistochemistry, a 1:200 diluted anti-FOXP3 monoclonal antibody (mAb) (Abcam ab22510), a 1:200 diluted anti-CD4 mAb (clone 4B12, MBL, Nagoya, Japan), and a 1:200 diluted anti-CD8 mAb (clone C8/144B, Dakocytomation) were used. The slides were incubated with the primary Ab overnight at 4°C and incubated with the horseradish peroxidase-conjugated goat anti-mouse-Ig, followed by the DAB chromogen as a substrate. The sections were counterstained with Mayers hematoxylin (Wako, Richmond, VA) and mounted. $CD4^+$, $CD8^+$, and $FOXP3^+$ cells were counted in all the portal areas for each patient. The size of the portal area was measured by computerized calculation using ImageJ software (the image processing and analysis in Java [http://rsb.info.nih.gov/ij/]). The number of $CD4^+$, $CD8^+$, and $FOXP3^+$ cells/mm^2 was assessed, dividing the number of $CD4^+$, $CD8^+$, and $FOXP3^+$ cells, respectively, within each portal area by the size of each portal area.

For immunofluorescent staining of CD4 and FOXP3, the sections were incubated overnight with a 1:20 diluted anti-CD4 mAb (K0003-1B, MBL, Nagoya, Japan) and stained using phycoerythrin-conjugated streptavidin (Vector Laborato-

ries, Burlingame, CA). The sections were further incubated with a 1:150 diluted fluorescein isothiocyanate-conjugated anti-FOXP3 mAb (Bioscience, San Diego, CA) at room temperature for 1 hr. For immunofluorescent staining of CD8 and FOXP3, the sections stained with the anti-FOXP3 mAb were further incubated with a 1:50 diluted phycoerythrin-conjugated anti-CD8 mAb (Bioscience, San Diego, CA) for 1 hr. The immunofluorescent stainings for CD4 or CD8, versus FOXP3 were overlaid as described previously (*11*).

The Reintroduction or the Increase of Immunosuppression

IS was reintroduced in IS free recipients (tolerance group [Gr-Tol]) or increased in the recipients in the weaning process, in case that advanced fibrosis (bridging fibrosis) was observed by single biopsy or progression of fibrosis was proven by repeated biopsy.

Statistical Analysis

All data were expressed by mean±SD. Data analyses were performed by one-way analysis of variance, Fisher's protected least significant difference test, Kruskal-Wallis test, Chi-square test, or Spearman's correlation test. *P* value less than 0.05 was considered to be significant. Statview-J5.0 software on Windows Xp (SAS Institute, Cary, NC) was used.

RESULTS

Characteristics of the Patients

The characteristics of the patients in Gr-Tol and maintenance IS group (Gr-IS) are listed in Table 1. The gender was comparable between Gr-Tol and Gr-IS (NS). Primary diseases included biliary atresia, Wilson's disease, fulminant hepatic failure, and so on in both groups. There was no difference between the two groups with respect to ABO compatibility. Donor age at LDLT did not differ between the two groups (Gr-Tol and Gr-IS: 32.7±6.1 years and 34.5±6.4 years; NS), whereas recipient age at LDLT in Gr-Tol was significantly younger than that in Gr-IS (Gr-Tol and Gr-IS: 1.0±1.7 years and 4.2±4.5 years; $P<0.005$). Serum alanine aminotransferase, aspartate aminotransferase, and total bilirubin levels at biopsy were comparable among tolerance, maintenance IS and baseline groups (Gr-BS) (each parameter, NS). γ-glutamyl transpeptidase level was significantly higher in Gr-BS, compared with those in the other two groups (Gr-Tol, Gr-IS vs. Gr-BS; $P<0.01$), but it was comparable between Gr-Tol and Gr-IS (NS). The interval between LDLT and biopsy, and this interval plus the donor age were significantly longer in Gr-Tol than those in Gr-IS (Gr-Tol and Gr-IS: 121.2±42.8 months and 52.2±22.4 months; $P<0.01$. 43.4±6.0 and 38.6±6.8 years; $P<0.01$). The number of examined portal areas per patient was comparable among the three groups (Gr-Tol, Gr-IS, and Gr-BS: 6.5, 7.7, and 7.5 [mean]; NS).

Study Limitation

Ideal controls for Gr-Tol would be arguably the patients on maintenance IS whose recipient age at LDLT and the interval between LDLT and biopsy are comparable with those in Gr-Tol. As described in the method, however, the patients who fulfilled the criteria invariably began weaning from IS

TABLE 1 Patients characteristics

	Gr-Tol (n=29)	Gr-IS (n=29)	Gr-BS (n=11)	*P*
Age (yr) at LDLT				
Recipient	1.0±1.7	4.2±4.5		<0.01
Donor	32.7±6.1	34.5±6.4		NS
Gender				
Male	7	12	NS	
Female	22	17		
ABO matching				
Identical	20	16		NS
Compatible	5	10		
Incompatible	4	3		
Immunosuppression at biopsy	None	Tacrolimus or CyA monotherapy, b.i.d.		
Interval during transplantation and biopsy (mo)	121.2±42.8	52.2±22.4		<0.01
Donor age plus interval between LDLT and biopsy (yr)	43.4±6.0	38.6±6.8		<0.01
AST (IU/L)	27.9±8.4	29.9±6.6	26.2±10.5	NS
ALT (IU/L)	24.2±12.7	20.0±6.5	36.3±19.7	NS
γ-GTP (IU/L)	21.1±10.6	19.8±10.0	44.8±30.9	NS (Gr-Tol vs. Gr-IS) <0.01 (Gr-Tol vs. Gr-BS) <0.01 (Gr-IS vs. Gr-BS)
T-Bil (mg/dL)	0.8±0.3	0.9±0.3	0.9±0.3	NS

Gr-Tol, tolerance group; Gr-IS, maintenance immunosuppression group; Gr-BS, baseline group; LDLT, living-donor liver transplantation; γ-GTP, γ-glutamyl transpeptidase; CyA, cyclosporine A.

following our elective protocol. Accordingly, the interval after LDLT in Gr-IS was significantly shorter than that in Gr-Tol. In addition, recipient age at LDLT was significantly younger in Gr-Tol than that in Gr-IS.

Lack of Typical Features of Acute and Chronic Rejection in Tolerant Patients

In the specimens of Gr-Tol, only slight cellular infiltrates were observed in the portal area, whereas the subendothelial spaces of the portal veins and the terminal hepatic venules, and the bile duct epitheliums were intact. The features of chronic rejection, such as the bile duct loss and centrilobular necroinflammation, were not observed in any specimen of Gr-Tol. Thus, Gr-Tol did not exhibit any typical feature of acute or chronic rejection.

The High Score of Graft Fibrosis in Tolerant Patients

The score of graft fibrosis in Gr-Tol was significantly higher than that in Gr-IS (Gr-Tol and Gr-IS (mean±SD): 1.6±1.2 and 0.9±0.8: $P<0.01$) (Fig. 1A, B). The score was significantly higher in Gr-IS than that in Gr-BS (Gr-IS and Gr-BS (mean±SD): 0.9±0.8 and 0.0±0.0; $P<0.01$) (Fig. 1A).

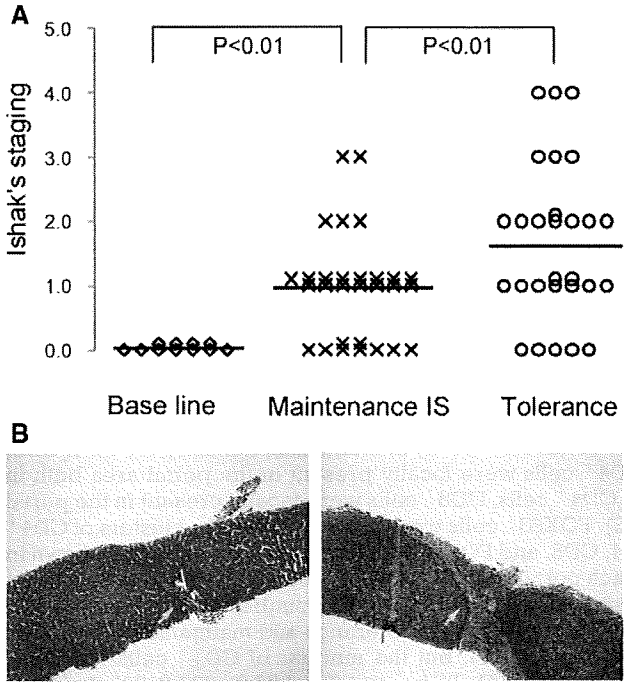

FIGURE 1. Graft fibrosis in tolerant grafts. (A) The degree of fibrosis was quantified by the Ishak's modified staging on Masson trichrome stained sections. The score in tolerance group was significantly higher than that in maintenance IS group. The score in maintenance IS group was significantly higher than that in baseline group (Gr-Tol, Gr-IS, and Gr-BS [mean±SD]: 1.6±1.2, 0.9±0.8, and 0.0±0.0; Gr-Tol vs. Gr-IS $P<0.01$, Gr-IS vs. Gr-BS $P<0.01$). The bar indicates mean values. (B) The score of tolerance group ranged F0 to F4. In a subset of tolerant grafts, bridging fibrosis was observed, whereas some tolerant grafts were normal. A representative picture of F0 is shown in the *left* and a representative picture of F4 is shown in the right. In the *left*, the *arrow* shows a normal portal area, whereas in the *right*, the *arrow* shows bridging fibrosis.

The Numbers of Graft Infiltrating CD4 Positive ($CD4^+$) and CD8 Positive ($CD8^+$) Cells

In tolerant grafts, CD4 positive ($CD4^+$) cells and CD8 positive ($CD8^+$) cells were focally present in the portal areas, but they were hardly detectable within the parenchyma (Fig. 2A, B). Like tolerant graft, $CD4^+$ and $CD8^+$ cells were present in the portal areas of grafts in the patients who were receiving maintenance IS. The numbers of $CD4^+$ cells in the transplanted liver was significantly increased, compared with that in the normal liver, whereas the numbers of $CD8^+$ cells in the transplanted liver was comparable with that in the normal liver. However, both the numbers of $CD4^+$ cells and $CD8^+$ cells were equivalent between tolerant and immunosuppressed grafts ($CD4^+$ cells; Gr-Tol, Gr-IS and Gr-BS [mean±SD]: 396.9±337.8, 320.8±238.1, and 93.3±98.8/mm^2, Gr-Tol vs. Gr-BS $P<0.01$, Gr-IS vs. Gr-BS $P<0.05$, Gr-Tol vs. Gr-IS NS; $CD8^+$ cells; Gr-Tol, Gr-IS and Gr-BS [mean±SD]: 1179.5±699.4, 1346.2±688.1, and 871.0±390.0 /mm^2, Gr-Tol vs. Gr-IS, Gr-BS NS) (Fig. 2D, E).

The number of FOXP3 Expressing ($FOXP3^+$) cells and the Degree of Fibrosis

$FOXP3^+$ cells were present within the clusters of $CD4^+$ and $CD8^+$ cells in the portal areas of the transplanted liver (Fig. 2C). The number of $FOXP3^+$ cells detected in tolerant grafts was significantly increased, compared with those in immunosuppressed grafts and the normal liver (Gr-Tol, Gr-IS, and Gr-BS [mean±SD]: 51.5±69.7, 23.3±31.1, and 6.8±7.9/mm^2, Gr-Tol vs. Gr-IS $P<0.05$, Gr-Tol vs. Gr-BS $P<0.05$, Gr-IS vs. Gr-BS) (Fig. 2F).

Nine biopsy samples from Gr-Tol were used for immunofluorescent staining. 5.1 $FOXP3^+$ cells/sample (between 1 and 12 cells) was detected in each slide of the nine samples. In total, 46 $FOXP3^+$ cells were observed in all of the nine samples analyzed. The overlay of immunofluorescent staining for CD4 or CD8, versus FOXP3 revealed that most of $FOXP3^+$ cells (92%) were $CD4^+$, whereas a small proportion of $FOXP3^+$ cells (8%) were $CD8^+$ (Fig. 2G). This supported the possibility that $CD4^+FOXP3^+$ regulatory T cells play an important role in the development of tolerance by suppressing other T cells within grafts in situ, which were reactive to donor antigens.

However, the number of $FOXP3^+$ cells did not correlate with the degree of fibrosis in tolerant or immunosuppressed grafts (Gr-Tol: $r=-0.21$, $r^2=0.0457$, $P=0.3$; Gr-IS: $r=0.17$, $r^2=0.03$, $P=0.4$) (Fig. 2H).

Time After Cessation of Immunosuppression Did Not Correlate With the Degree of Fibrosis

As shown in Figure 3(A), in Gr-Tol, time after cessation of IS did not correlate with the degree of fibrosis ($r=-0.001$, $r^2=0.000001$, $P=0.996$).

Neither the recipient age at LDLT nor the donor age plus the interval between LDLT and biopsy correlated with the degree of fibrosis.

Recipient age at LDLT was significantly younger and donor age plus the interval between LDLT and biopsy was

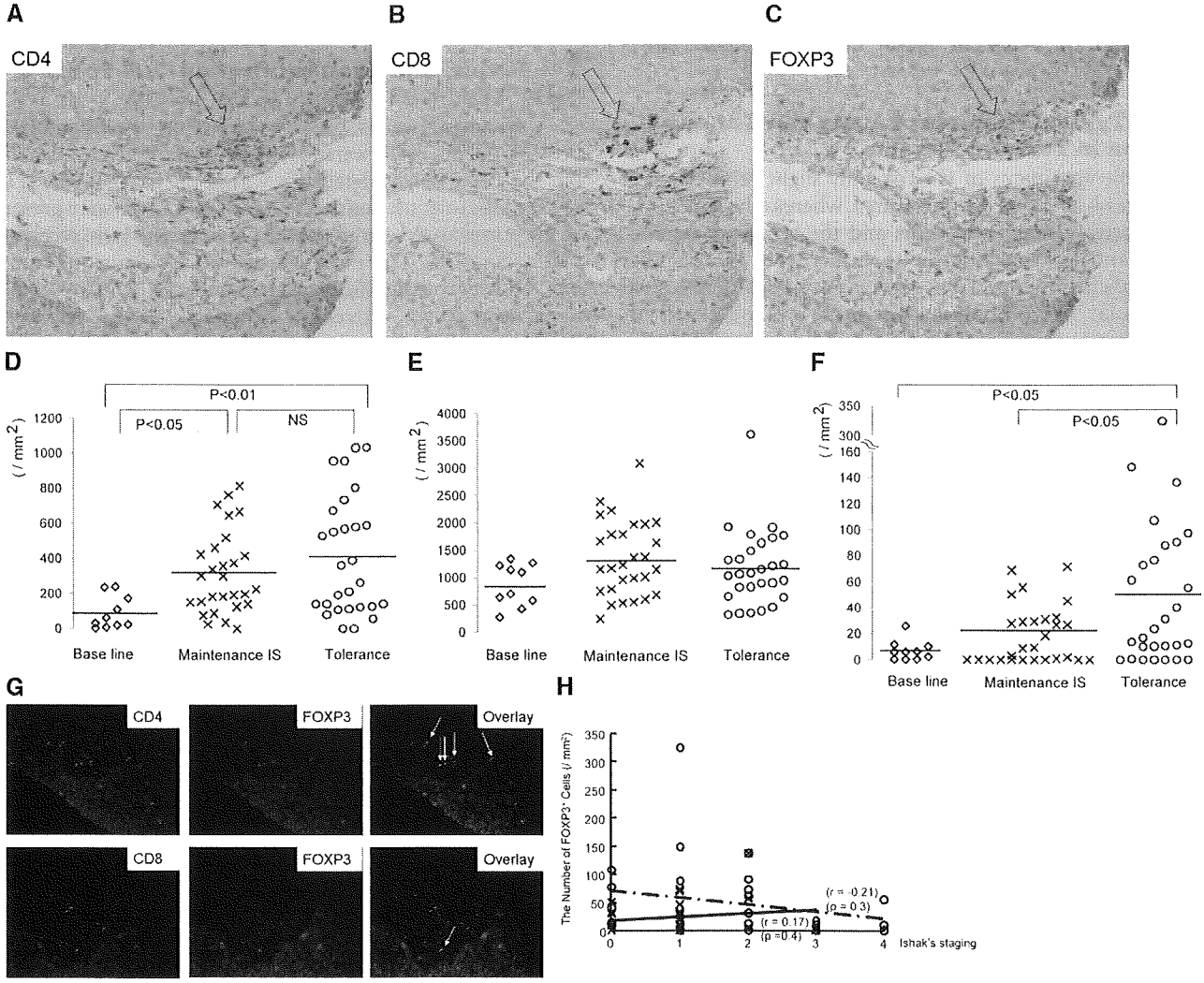

FIGURE 2. Graft infiltrating CD4$^+$, CD8$^+$, and FOXP3$^+$ cells in tolerant and immunosuppressed grafts and lack of the correlation between the number of FOXP3$^+$ cells and the degree of fibrosis. (A–C) Immunohistochemistry for CD4, CD8, and FOXP3. Immunohistochemistry was performed for CD4. CD4$^+$ cells were focally present in the portal area both in tolerance (Gr-Tol) and maintenance IS groups (Gr-IS). Similar to CD4$^+$ cells, CD8$^+$ cells were focally present in the portal area both in tolerance (Gr-Tol) and maintenance IS groups (Gr-IS). FOXP3$^+$ cells were present within the clusters of CD4$^+$ and CD8$^+$ cells in the portal area. Representative pictures of CD4, CD8, and FOXP3 staining of tolerant grafts are shown in (A), (B), and (C). (D) The number of CD4$^+$ cells was counted for each portal area. The size of each portal area was measured by computerized calculation. The number of CD4$^+$ cells/mm^2 was assessed by dividing the number of CD4$^+$ cells within each portal area by the size of each portal area. The numbers of CD4$^+$ cells/mm^2 in tolerance and maintenance IS groups were significantly increased, compared with that in baseline group (Gr-BS), but the number of CD4$^+$ cells/mm^2 was equivalent between tolerance and maintenance IS groups (Gr-Tol, Gr-IS, and Gr-BS [mean±SD]: 396.9±337.8, 320.8±238.1, and 93.3±98.8/mm^2, Gr-Tol vs. Gr-BS $P<0.01$, Gr-IS vs. Gr-BS $P<0.05$, Gr-Tol vs. Gr-IS NS). The bar indicates mean values. (E) The number of CD8$^+$ cells/mm^2 was assessed in the same manner as CD4$^+$ cells. Unlike CD4$^+$ cells, the number of CD8$^+$ cells/mm^2 did not differ among tolerance, maintenance IS, and baseline groups (Gr-Tol, Gr-IS, and Gr-BS [mean±SD]: 1179.5±699.4, 1346.2±688.1, and 871.0±390.0/mm^2, Gr-Tol versus Gr-IS, Gr -BS NS). The bar indicates mean values. (F) The number of FOXP3$^+$ cells. Although the number of CD4$^+$ and CD8$^+$ cells were equivalent between tolerance and maintenance IS groups, the number of FOXP3$^+$ cells/mm^2 was significantly increased in tolerance group (Gr-Tol), compared with those in maintenance IS (Gr-IS) and baseline groups(Gr-BS) (Gr-Tol, Gr-IS, and Gr-BS [mean±SD]: 51.5±69.7, 23.3±31.1, and 6.8±7.9/mm^2, Gr-Tol vs. Gr-BS $P<0.01$, Gr-IS vs. Gr-BS $P<0.05$, Gr-Tol vs. Gr-IS NS). The bar indicates mean values. (G) Immunofluorescent staining for CD4 versus FOXP3 and CD8 versus FOXP3 in tolerance group. Representative pictures of immunofluorescent staining for CD4 versus FOXP3 and CD8 versus FOXP3 in nine tolerance grafts are shown. 5.1 FOXP3$^+$ cells/sample (between 1 and 12 cells) were detected in each slide of the nine samples. In total, 46 FOXP3$^+$ cells were observed in all of the nine samples analyzed. Staining for CD4 (red, *upper left*) and FOXP3 (green, *upper middle*). Double-positive cells are seen as greenish-yellow (*upper right*, indicated by the *arrows*). Staining for CD8 (red, *lower left*) and FOXP3 (Green, *lower middle*). Double-positive cells are seen as greenish-yellow (*upper right*, indicated by the *arrows*). The overlay of immunofluorescent staining for CD4 or CD8, versus FOXP3 revealed that most of FOXP3$^+$ cells (92%) were CD4$^+$,

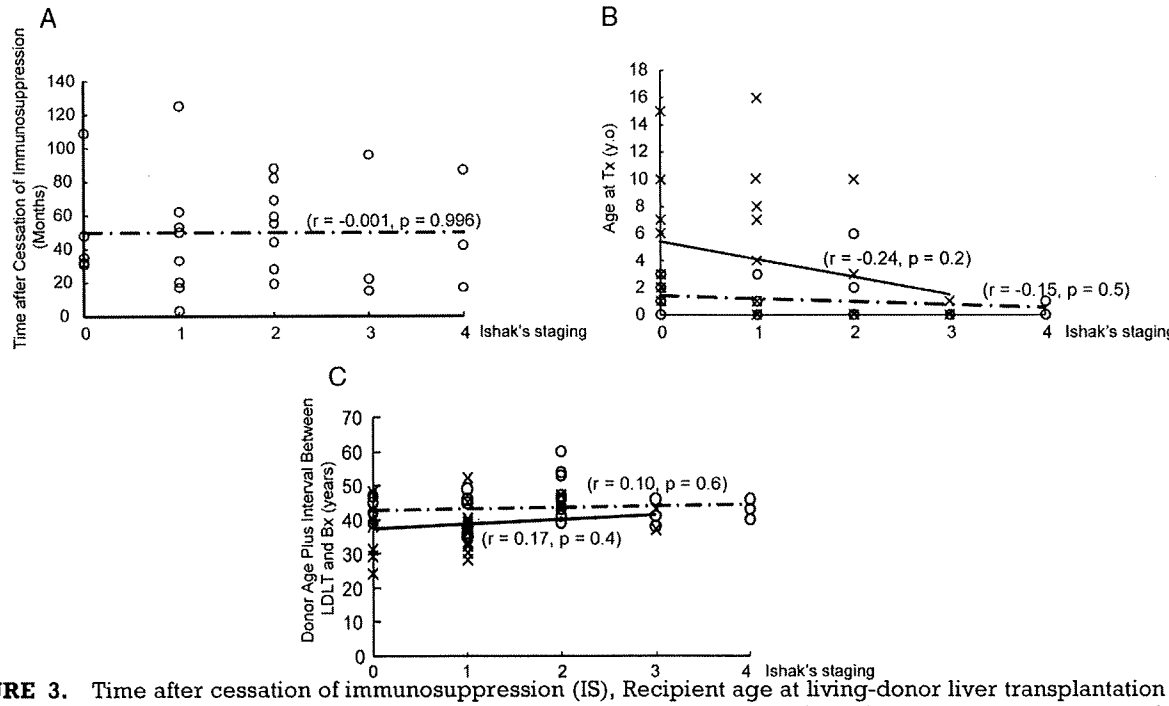

FIGURE 3. Time after cessation of immunosuppression (IS), Recipient age at living-donor liver transplantation (LDLT), and donor age plus the interval between LDLT and biopsy versus the degree of fibrosis. (A) In tolerance group, time after cessation of IS did not correlate with the degree of fibrosis (Gr-Tol: $r=-0.001$, $r^2=0.000001$, $P=0.996$). (B) The recipients age at LDLT did not correlate with the degree of fibrosis in tolerance group or maintenance IS group (Gr-Tol and Gr-IS: $r=-0.24$, $r^2=0.021$, $P=0.2$ and $r=-0.15$ $r^2=0.059$, $P=0.5$). (C) The donor age plus the interval between LDLT and biopsy did not correlate with the degree of fibrosis in tolerance group or maintenance IS group (Gr-Tol and Gr-IS: $r=0.1$, $r^2=0.009$, $P=0.6$ and $r=0.17$, $r^2=0.0294$, $P=0.4$). ○ and the *dashed line* indicate tolerance group and × and the *solid line* indicate maintenance IS group.

significantly longer in Gr-Tol than those in Gr-IS. One may hypothesize that the growth and the aging of grafts may cause fibrosis in an antigen-independent fashion. To examine these possibilities, whether or not recipient age at LDLT or donor age plus the interval between LDLT and biopsy would correlate with the degree of fibrosis was evaluated. As shown in Figure 3B, recipient age at LDLT or donor age plus the interval between LDLT and biopsy did not correlate with the degree of fibrosis in Gr-Tol or in Gr-IS (recipient age at LDLT, Gr-Tol, and Gr-IS: $r=-0.24$, $r^2=0.021$, $P=0.2$ and $r=-0.15$, $r=0.059$, $P=0.5$, donor age plus the interval between LDLT and biopsy, Gr-Tol and Gr-IS: $r=0.1$, $r^2=0.009$, $P=0.6$ and $r=0.17$, $r^2=0.0294$, $P=0.4$).

An Improvement of Fibrosis After the Reintroduction or the Increase of Immunosuppression

Because it remains elusive whether fibrosis in Gr-Tol is antigen dependent, IS was reintroduced in the IS free recipients or increased in the recipients in the weaning process, in case that advanced fibrosis (bridging fibrosis) was observed by single biopsy or progression of fibrosis was proven by repeated biopsy. IS was reintroduced in four IS free recipients and increased in seven recipients in the weaning process and the changes of the fibrosis was examined by follow-up biopsy. IS was reintroduced or increased due to bridging fibrosis in six patients or because of progression of fibrosis in five patients (Table 2). The follow-up biopsy was performed a mean of 14 months (ranging from 4 to 54 months) after the previous biopsy (Table 2). As shown in Figure 4, an improvement of fibrosis was observed in seven patients and there was no change of fibrosis in four patients.

DISCUSSION

Does Operational Tolerance Offer Protection Against Antigen-Dependent Histologic Changes of Grafts?

Clinical operational tolerance is defined as a stable normal graft function in the total absence of a requirement for maintenance IS (*3, 4*). In reviewing literatures, only five centers have published articles on operational tolerance in liver transplantation (*6, 12–17*). However, no biopsy has been performed in those tolerant patients. Accordingly, there have been no strictly defined histologic criteria of operational tol-

FIGURE 2. (CONTINUED) whereas a small proportion of $FOXP3^+$ cells (8%) were $CD8^+$. (H) The correlation between the number of $FOXP3^+$ cells and the degree of fibrosis. The number of $FOXP3^+$ cells/mm^2 did not correlate with the degree of the fibrosis in tolerance or maintenance IS groups (Gr-Tol: $r=-0.21$, $r^2=0.0457$, $P=0.3$; Gr-IS: $r=0.17$, $r^2=0.03$, $P=0.4$). ○ and the *dashed line* indicate tolerance group and × and the solid line indicate maintenance IS group.

TABLE 2 The reintroduction or the increase of immunosuppression because of bridging fibrosis or progression of fibrosis

Patient number	IS	Ishak's stage	Reason of reintroduction or an increase of IS	Time between previous and follow-up biopsy (mo)
27	Off→1/d	4	Bridging fibrosis	4
119	Off→2/d	2	Progression of fibrosis	11
171	1/mo→1/wk	3	Bridging fibrosis	7
190	Off→1/d	1	Progression of fibrosis	16
191	1/mo→1/d	2	Progression of fibrosis	12
192	Off→1/d	3	Bridging fibrosis	12
204	1/wk→1/d	2	Progression of fibrosis	12
216	1/wk→1/d	4	Bridging fibrosis	19
247	1/d→2/d	4	Bridging fibrosis	6
291	1/wk→2/d	3	Bridging fibrosis	54
344	3/wk→1/d	2	Progression of fibrosis	10
				14 (mean)

IS, immunosuppression.

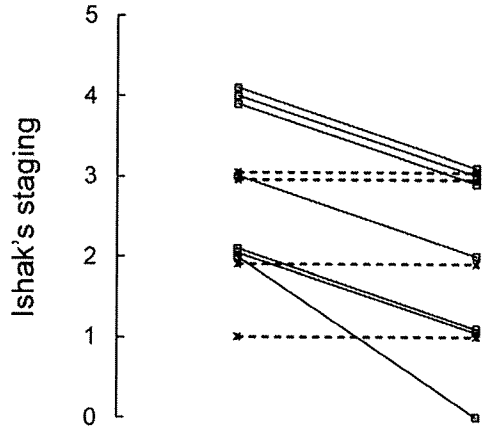

FIGURE 4. Changes of fibrosis after the reintroduction or the increase of immunosuppressant. In 11 patients, immunosuppressant was reintroduced or increased due to the presence of bridging fibrosis or progression of fibrosis. Seven patients (*solid lines*) exhibited the improvement of the fibrosis, whereas four patients (*dashed lines*) exhibited no change of the fibrosis, a mean of 14 months after the reintroduction or the increase of immunosuppression.

erance to date. Thus, a question arose as to whether the establishment of operational tolerance, a phenomenon equivalent to protection against clinically manifest rejection (the presence of a normal liver test), similarly offers protection against antigen-dependent histologic changes of grafts characterized by cellular infiltrates, vessels damages, fibrosis, and changes of the bile ducts. To address this question, protocol biopsy was performed on operationally tolerant patients, and histology of accepted grafts was studied in detail.

Graft Fibrosis Was Observed in Grafts in a Subset of Tolerant Patients

In our study, the evaluation of conventional histology in our tolerant patients demonstrated that significant infiltrates were not detectable within the subendothelial spaces of the portal veins or the terminal hepatic venules, or within bile ducts. In addition, neither a loss of the bile duct nor centrilobular necroinflammation was observed on CK7 stained or hematoxylin and eosin stained sections. Thus, following the Banff schema, the criteria of acute or chronic rejection were not met in tolerant grafts. However, fibrosis was observed on Masson trichrome stained section to a greater extent in a subset of the patients in Gr-Tol than that in Gr-IS. To decide whether IS must be reintroduced in the IS free recipients who exhibited advanced fibrosis, it is of paramount importance to elucidate whether fibrosis observed in Gr-Tol is antigen dependent or independent (*18*).

Is the Fibrosis Observed in Tolerant Grafts Antigen-Dependent?

Once some researchers have assumed that the development of tolerance is accompanied by normal graft histology indistinguishable from histology of syngeneic grafts in experimental transplantation models (*19–24*). On the other hand, it seems that a frequent pathologic feature of grafts in tolerant recipients is the presence of mononuclear cell infiltrates (*25–28*). In accordance with such experimental models (*25–28*), our immunohistochemical analyses demonstrated that $CD4^+$ cells were unequivocally present within tolerant grafts. To predict long-term outcome of tolerant grafts, it is important to elucidate whether those infiltrates are indication of an immunoregulatory process that maintains graft acceptance or a reflection of an ongoing low grade destructive immune response. To address this question, the expression of FOXP3 (a specific marker of regulatory T cells) was examined (*29*). In tolerant grafts, $FOXP3^+$ cells were present within the cluster of $CD4^+$ cells and most of them were $CD4^+$. In addition, the number of $FOXP3^+$ cells was significantly increased in Gr-Tol, compared with that in Gr-IS, although the total number of $CD4^+$ cells in grafts was equivalent between Gr-Tol and Gr-IS. We and others have shown previously that up-regulation in the frequency of $CD4^+CD25^{high+}$ T cells persists in the peripheral blood of tolerant liver transplantation recipients (*30, 31*). Additionally, our previous in vitro functional study demonstrated that $CD4^+CD25^{high+}$ T cells in the peripheral blood of tolerant LDLT recipients exerted their suppressive property against other T cells responding to donor alloantigens (*32*). Collectively, these data support the hypothesis that $FOXP3^+CD4^+$ cells identified within tolerant grafts may facilitate the development of unresponsiveness to donor alloantigens by protecting grafts against rejection in situ. However, there was no correlation between the number of $FOXP3^+$ cells and the degree of fibrosis.

In addition, time after cessation of IS (the length of IS free period) did not correlate with the degree of fibrosis in Gr-Tol. Thus, these data did not support the possibility that fibrosis in Gr-Tol was antigen dependent.

Is the Fibrosis Observed in Tolerant Grafts Antigen-Independent?

As described in the results, the interval between LDLT and biopsy in Gr-Tol was significantly longer than that in Gr-IS. Because the donor age was comparable between tolerance and Gr-ISs, the donor age plus the interval between LDLT and biopsy was longer in Gr-Tol than that in Gr-IS. McLean et al. (*33*) demonstrated that subsinusoidal fibrosis occurs as a consequence of liver aging. Thus, there was one possibility that the fibrosis in the tolerant patients was solely a consequence of graft aging. In addition, the ages at LDLT of the recipients who could successfully stop IS were significantly younger that those of recipients who were still taking maintenance IS. Living donor and "split" liver grafts show more structural abnormalities including fibrosis than whole cadaveric liver grafts with time post-Tx (Anthony J. Demetris, personal communication). The growth of grafts can offer some physical stress to the grafts themselves in living donor and split grafts and such stress can cause fibrosis. In theory, grafts in Gr-Tol had to grow to a greater extent than grafts in Gr-IS. Therefore, the difference in fibrosis between Gr-Tol and Gr-IS might be caused by the difference in the extent of physical stress during the growth of grafts between the two groups. To examine these possibilities, it was evaluated whether there was a correlation between recipient age at LDLT or donor age plus the interval between LDLT and biopsy versus the degree of fibrosis. However, recipient age at LDLT or donor age plus the interval between LDLT and biopsy did not correlate with the degree of fibrosis. Hence, these data did not support the possibility that fibrosis in Gr-Tol was antigen independent. However, we must still pay some attention to the possibility that fibrosis observed in tolerant grafts was antigen independent. Hepatitis E virus (HEV), albeit having been responsible for acute hepatitis that does not progress to chronic hepatitis in normal individuals, could cause chronic hepatitis in immunosuppressed patients (*34*, *35*). Seroprevalence studies have reported that anti-HEV IgG antibodies are present in the peripheral blood in a high proportion (6%–16%) of renal transplantation recipients (*36*, *37*). Because the degree of fibrosis was significantly higher even in the patients on maintenance IS than that in the normal liver base line group (Fig 1A), we are now examining the possibility that HEV infection would persist and cause the development of the fibrosis in the presence of IS.

The Reintroduction or the Increase of IS Improved the Fibrosis. This Supports the Possibility That Fibrosis Observed in Tolerant Graft is Antigen Dependent

Because our investigation had not elucidate whether fibrosis observed in tolerant grafts was antigen dependent or independent, IS was reintroduced or increased in the tolerant recipients or the recipients in the weaning process in case that single biopsy demonstrated bridging fibrosis or repeated biopsy demonstrated apparent progression of fibrosis. It should be noted that after the reintroduction or the increase of IS the degree of fibrosis was improved in a subset of the patients. This finding supported the hypothesis that fibrosis observed in tolerant graft was antigen dependent. However, because the number of patients in this setting was not large enough and the improvement of fibrosis was not dramatic, further observation is required to validate this conclusion as from the dataset presented here. There may be significant sampling problems associated with living donor grafts that have to be taken into consideration. If a biopsy sample is taken near the cutting edge of the liver or from an area that has suboptimal blood inflow, or blood or bile outflow, the liver graft morphology can be significantly altered. At least, we can not entirely exclude the possibility that the first biopsy sample was taken from such areas of more antigen-independent damage and the subsequent biopsy sample was taken from areas of less damage or intact areas.

Conclusion

This is the first report providing detailed evidence that a subset of operationally tolerant patients, despite a normal or a quasi-normal liver test, exhibited graft fibrosis. It remains elusive whether fibrosis observed in tolerant graft is antigen dependent or independent. Our finding that after the reintroduction or the increase of IS in the IS free recipients or the recipients in the weaning process, the degree of fibrosis was improved supported the hypothesis that fibrosis observed in tolerant grafts was antigen dependent.

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Value of FOXP3 Expression in Peripheral Blood as Rejection Marker After Miniature Swine Lung Transplantation

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Background: Outcome for highly immunogenic lung transplantation remains unsatisfactory despite the development of potent immunosuppressants. The poor outcome may be the result of a lack of minimally invasive methods to detect early rejection. There is emerging clinical evidence that, paradoxically, expression of forkhead box P3 (FOXP3, a specific marker for the regulatory T cells) is upregulated within rejecting grafts.

Methods: Orthotopic lung transplantation was performed using miniature swine without immunosuppression. Rejection was monitored by chest radiography and open lung biopsy. Expressions levels of *FOXP3*, *perforin*, *Fas-L* and *IP-10* mRNA were quantified in the peripheral blood. In addition, rescue immunosuppressive therapy (steroid plus tacrolimus) was administered on post-operative day (POD) 4 or 6.

Results: Early rejection was detected by open lung biopsy, but misdiagnosed by chest radiography on POD 4. Expression of *FOXP3* in the peripheral blood reached its highest value as early as POD 4, followed by a decline. Such an increase of *FOXP3* was not observed in recipients given high-dose tacrolimus. Neither *perforin*, *Fas-L* or *IP-10* in the peripheral blood exhibited significant fluctuations in the early phase of rejection. Rescue immunosuppressive therapy from POD 4, when peak *FOXP3* was seen, prolonged graft survival (27.2 days, versus 9.1 days without immunosuppression, $p < 0.001$), in contrast to POD 6, when rejection was suspected by chest radiography (11.5 days, p = not statistically significant [NS]).

Conclusions: In a miniature swine lung transplantation model, the *FOXP3* mRNA level in the peripheral blood was upregulated at an early phase of rejection. The clinical implication of this finding remains to be elucidated. J Heart Lung Transplant 2008;27:1293-301. Copyright © 2008 by the International Society for Heart and Lung Transplantation.

In the era of potent immunosuppressants, the incidence of acute rejection has been dramatically reduced after clinical organ transplants.[1] The 5-year survival rate has reached 70% to 80% for most solid organ transplants.[2-4] Unfortunately, improvement in survival rate for lung transplantation has not been as dramatic as compared with other organs, with a 5-year survival rate of <50%.[5] This is due to the fact that the lung, which is the most immunogenic organ to transplant, can generate extremely vigorous rejection.[6,7] Nevertheless, a reliable, non-invasive method to detect early rejection has not been established,[8-12] although some approaches have been proposed.[13,14] In clinical lung transplantation, profound immunosuppression is uniformly administered, taking precautions against rejection with attending complications.[5] Infections represent the leading cause of death in the early post-lung transplantation period.[5] If a reliable, non-invasive method becomes available, then clinicians could decrease the baseline immunosuppression and better tailor the immunosuppression for the individual patient, which may decrease the rate of death from infections.

Studies in mice demonstrated that forkhead box P3 (Foxp3) was a master control gene, exclusively ex-

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pressed by regulatory T cells (Tregs) (i.e., not expressed by non-Tregs).[15-17] In some rodent transplant models, high levels of *Foxp3* mRNA and a substantial number of Foxp3 protein-expressing cells were found in tolerant grafts, but not in rejecting grafts.[18-20] Thus, in rodents, *Foxp3* mRNA and Foxp3 protein may be reliable biomarkers of tolerance. However, it has been reported that *FOXP3* mRNA and protein levels are paradoxically increased within rejecting cardiac grafts in humans or grafts and urine specimens in renal allograft recipients.[21-24] Therefore, the question arises as to whether FOXP3 is a biomarker of tolerance, or whether FOXP3 can also be useful to detect rejection.

We quantified the *FOXP3* mRNA level in the peripheral blood during acute rejection using a miniature pig lung transplantation model. The *FOXP3* mRNA level in the peripheral blood was upregulated at an early phase of rejection. It must be determined whether measurement of peripheral blood FOXP3 level would be useful as a minimally invasive method to detect the early phase of rejection in clinical lung transplantation.

METHODS

Animals

Male or female swine leukocyte antigen (SLA)-unknown Clawn miniature swine (22 to 30 kg, 6 to 10 months old), were purchased from the Japan Farm CLAWN Institute (Kagoshima, Japan). The pigs were raised in conventional housing facilities and free of foot-and-mouth disease, hog cholera, vesicular disease, African swine fever and hepatitis E. All animal care and procedures were in compliance with the "Principles of Animal Care," formulated by the animal facility of Kyoto University.

Experimental Groups

Five groups were studied that were differentiated according to the type of operation and whether and when immunosuppressants were administered:

Immunosuppression-free rejection group (n = 10): Lung transplantation, no immunosuppression.

Clamp reperfusion group (n = 4): Clamp reperfusion, no transplantation, no immunosuppression.

Induction immunosuppression group (n = 3): Lung transplantation, high-dose tacrolimus from post-operative day (POD) 1 to 12.

POD 4 rescue immunosuppressive therapy group (n = 6): Lung transplantation, rescue immunosuppressive therapy from PODs 4 to 9.

POD 6 rescue immunosuppressive therapy group (n = 4): Lung transplantation, rescue immunosuppressive therapy from PODs 6 to 11.

Isolation of PBMCs

Blood samples were collected in heparinized test tubes. The peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation.[25]

Mixed Lymphocyte Culture

For responders, $CD4^+$ cells were positively selected on a LS separation column by the MACS system (magnetic cell sorting; Miltenyi Biotech).[26] The medium used for culture was RPMI with 10% fetal calf serum and 2-mercaptoethanol. Cells were seeded in 96-well microtiter plates in replicates of four at a concentration of 5×10^4 cells/well, 1×10^5 cells/well-irradiated (25-Gy) stimulator PBMCs were added to the culture for 96 hours, and ^{3}H-thymidine uptake was determined using standard techniques.[27] For lung transplantation, reactive donor-recipient pairs were used. Reactivity was defined as a tritiated thymidine incorporation value (cpm) of >5,000.

Lung Transplantation

Orthotopic left lung transplantation was performed as previously described.[28,29] ET-Kyoto solution (Otsuka Pharmaceutical, Tokushima, Japan [not for sale]) was used to flush and preserve the graft.[30] During the operation, two catheters were placed into the bilateral external jugular veins. One catheter was used to administer fluid, antibiotics and immunosuppressants, and the other was to obtain blood.

Clamp Reperfusion Model

After heparinization (300 U/kg), the vessels of the left lung were clamped. After the pulmonary artery was catheterized and the pulmonary vein was incised for fluid drainage, the lung was topically cooled with iced saline and flushed in situ with 4 liters of cold ET-Kyoto solution. The lung was left in situ and cooled for 1.5 hours (comparable to the cold ischemic time of lung transplantation) with iced saline. The iced saline was then removed and the lung was kept at room temperature for 1.5 hours (comparable to the warm ischemic time of lung transplantation). During this time, the catheter for the artery was removed and the incisions in the artery and the vein were closed by sutures. Finally, the vessels were declamped and the lung was reperfused.[31]

Monitoring Rejection

Chest radiography was performed on POD 3 to 7, and 10. The findings were scored on a grade of 0 to 4, based on our grading system (Table 1). Under general anesthesia, open lung biopsy was performed on PODs 4, 7 and 10. The specimens were evaluated using conven-