AMAの出現に何らかの関与を示唆しているが，PBC特異性に関してはいまだ問題が残っているようである．

2．胆汁中および胆管上皮に存在する菌体成分

Ideら[25]は，PBC患者血清中に存在するIgM型抗リピドA（LPSの活性部位）抗体について検討しており，PBCでは対照群に比しIgM型抗リピドA抗体の保有率が有意に高く，さらにUDCA治療にてこの抗体価も減少すると報告している．また，PBC肝組織内におけるLPSの組織内分布も検討し，肝細胞，クッパー細胞のみならず胆管にもLPSが存在していること，さらにUDCA投与により胆管でのLPS発現が減弱することも報告している[26]．また，胆道感染症のみならずPBCや他の肝胆道系疾患でも，胆汁中にエンドトキシン（LPS）やグラム陽性菌の主要壁成分であるリポタイコ酸が存在しており[27-29]，下記胆管自然免疫のPAMP（Pathogen-associated molecular pattern；宿主免疫活性化作用をもつ微生物成分）として作用する．

3．胆汁および肝組織中の細菌遺伝子

われわれは，胆汁から細菌16S rRNA遺伝子をPCR法で増幅後，配列解読により菌種を同定した結果，PBCの胆汁中には対照疾患に比べグラム陽性球菌が優位に見られることを報告した[28]．また，Nilssonら[30,31]は，PBC，PSC，非胆汁うっ滞性肝硬変，正常肝の肝組織を対象に，ヘリコバクター属および*H.pylori*，*H.bilis*，*H.pullorum*，*H.hepaticus*に特異的なプライマーを設定しPCR法にて検出したところ，PBCやPSCなどの慢性胆汁うっ滞性肝疾患の80～90％にヘリコバクター属が検出され，さらにそれらのほとんどが*H.pylori*も検出されたと報告している．また，PBC患者血清中の抗PDC抗体と抗*H.pylori*抗体とがよく相関することから，*H.pylori*がPBCの自己免疫現象に関わっていると考えられる[32]．しかし，Tanakaら[33]の報告ではPBC 29例中1例しか*H.pylori*が検出されず，またわれわれの検討でも*H.pylori*の関与は否定的な結果を得ている．

4．類上皮肉芽腫と細菌

類上皮肉芽腫はPBC症例の80％前後に出現し[34]，特に障害胆管周囲にみられる場合，肉芽腫性胆管炎と呼称され診断価値の高い組織所見である（図1）．一般病理学的に肉芽腫は感染や異物に対する生体反応として捉えられており，PBCの肉芽腫形成に何らかの感染症あるいは菌体成分（特に結核菌などの抗酸菌）の関与が示唆される．近年，Mycobacteriumの遺伝子がクローン病の病変部（腸管壁や所属リンパ節）から分子生物学的に検出され，病態形成との関連性が示唆されている．Vilagutら[35]は肉芽腫形成を特徴とするMycobacteriaに注目し，PBCの肝組織中から非定型的抗酸菌であるMycobacteria gordonaeのDNAが高率に検出されることを報告した．その後の追跡研究で，この菌種の65 kDa Heat shock proteinに対する抗体がPBC全例で検出され，さらにヒトPDC-E2やBCKD-E2と交差反応を示すことも証明した[36-38]．しかし，O'Donohueら[39]およびTanakaら[33]は否定的な成績を発表しており，PBC肝組織内からの抗酸菌の検出に関して一定の結論は得られていない．

われわれは，PBCの肉芽腫形成と細菌との直接的な関連性を検討するため，マイクロダイセクション法と分子生物学的検討により肉芽腫部に存在する菌種を同定した．その結果，種々の腸内細菌由来の遺伝子が検出されたが，*Propionibacterium acnes*（*P.acnes*）が主

図1　PBCの肉芽腫性胆管炎
慢性非化膿性破壊性胆管炎に隣接して肉芽腫形成(Gra)を認める.

たるクローンとして存在していることを報告し[40], *P.acnes* 菌に対する抗体を用いた免疫染色でも肉芽腫部に陽性所見が見られた(図2). *P.acnes* は全身性肉芽腫性疾患であるサルコイドーシスの病原菌としても注目されている菌種である[41]. *P.acnes* の異常な集積と同菌体に対する免疫反応がPBCの肉芽腫形成の主たる要因であると示唆されるが, 胆管炎などその他の病態形成における *P.acnes* の関与については不明である.

5. クラミジア感染症

2002年, Abdulkarimら[42,43]による米国消化器病学会およびEASLでの発表を機に, PBCとクラミジアとの関連性が注目されている. 彼らは, クラミジア肺炎抗原に対する抗体を用いて免疫組織化学的検討を行ったところ, PBC肝の全例(25例)およびPSCの39例中6例で肝細胞内に陽性所見を認めたが, アルコール性肝疾患(40例)やC型慢性肝炎(22例)では陰性であったと報告した. しかし, Leungら[44]は, ヒトに対して病原性を示す *C.pneumoniae*, *C.trachomatis* を抗原としてPBC患者血清と反応させた結果, AMA陽性のPBC血清のうち91%が陽性で, AMA陰性のPBC血清では21%, 対照群では5%以下であったが, PCRや免疫組織化学的手法を用いて肝組織中からはクラミジアを検出することはできなかったと報告しており, クラミジアとPBCとの関連性について疑問を投げかけた. クラミジアは性病性リンパ肉芽腫(LGV)の原因菌でもあり, 肉芽腫形成を起こし得る点でPBCと類似性がある. 今後, 追跡データの報告が待たれる領域である.

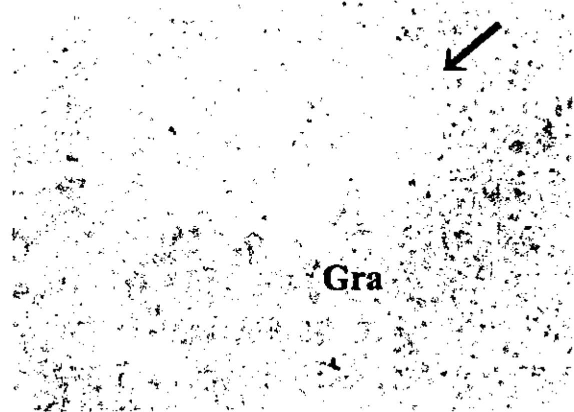

図2　*P.acnes* 菌体の免疫組織化学的検出
抗 *P.acnes* モノクローナル抗体(PAB抗体, 東京医科歯科大学 江石先生より分与)を用いた免疫組織化学的染色で, PBCの肉芽腫(Gra)に陽性所見を認める. 矢印は障害を伴った小葉間胆管.

6. ***Novosphingobium aromaticivorans* (*N.aromaticivorans*)**

Selmiら[45]は, 蛋白データベースよりヒトPDC-E2の抗原決定基領域に高いホモロジーを示すペプチドを検索し, ヒトPDC-E2に類似する2種の蛋白(47 kDaと50 kDa)を有する *N.aromaticivorans* に注目した. *N.aromaticivorans* は, 土壌など自然界に存在するグラム陰性好気性菌で, xenobioticな有機化合物やエストロゲンを代謝する環境菌である. PDC-E2抗体陽性のPBC血清すべてが *N.aromaticivorans* の47 kDa, 50 kD蛋白共に交差反応性を示し, さらに大腸菌に比し100～1,000倍の反応性を示した. また, 糞便中

からも *N.aromaticivorans* 由来の遺伝子が検出され，PBC原因菌の候補として報告している．

3 PBCの病因とウイルス感染症

PBC患者に出現するAMAのイムノグロブリン型はIgG3型とIgM型が主であり[46]，末梢血リンパ球からのIgG3分泌[47]，さらにPBC患者では血中IgG3濃度が健常人に比べ高いこと[48]が報告されている．IgG3は自己免疫疾患の誘因となり得るある種のウイルス感染症の際に認められることより，PBCとウイルス感染症との関連性が示唆される．また，非PBC症例の培養胆管細胞にPBC患者由来のリンパ節抽出液を添加すると，胆管細胞からPDC-E2，E3-binding protein（Protein X）が表出されることが報告されており，ウイルスや細菌を含め何らかの感染性因子の関与が示唆されている[49]．

1．βレトロウイルス

近年，PBCとレトロウイルスとの関連性が注目されている．1988年，Munozら[50]が，human immunodeficiency virus-1（HIV-1）に対する抗体がPBC患者血清中に存在していることを報告した．当初，擬陽性と考えられていたが，シェーグレン症候群やSLEなどの自己免疫性疾患でも同様な血清学的データが報告され[51,52]，自己免疫現象におけるHIV-1の関与が想定された．その後，1998年にMasonら[53]は，HIV-1のp24 gag蛋白およびHuman intracisternal A-type particle（HIAP）に対する抗体を調べた結果，p24 gag蛋白に対する抗体がPBCの35％，SLEの29％，健常者の4％で検出され，HIAPに対する抗体がPBCの51％，SLEの58％，健常者の4％で検出され，これらレトロウイルス蛋白をトリガーとした分子相同性による自己免疫反応がPBCを含めた自己免疫性疾患の病態に関与していることを示唆した．その後，Xuら[54,55]はPBC患者から得られたヒト胆管細胞のcDNAライブラリーより，mouse mammary tumor virus（MMTV）およびヒト乳癌組織よりクローニングされたβレトロウイルスと類似した塩基配列を有するcDNA断片を検出し，PBCのリンパ節におけるウイルス粒子の存在，さらにMMTVが正常胆管細胞からの自己抗原（PDC-E2）を表出させ得ることも証明している．現在のところ，PBCの原因としてのウイルス感染を示唆する最も有力な報告である．

2．その他のウイルス

Minukら[56]は，レオウイルス（Reovirus type 3）に対する血中抗体を検討し，PBCやPSCではその他の慢性肝疾患や健常人に比し抗体の陽性率が有意に高く，抗体価も高いが，肝生検材料を使った免疫組織化学的検討ではレオウイルスを証明できなかったと報告している．また，Morshedら[57]は，PBC患者の末梢単核球，肝組織，唾液からEpstein-Barr virus（EBV）をPCR法にて検出し，他の肝疾患群に比しPBCで有意に高率であることから，PBC患者ではEBV感染に対する免疫反応が低下しており，PBCの病態形成に関与している可能性を述べている．

4 胆管周囲の微小環境と胆管系自然免疫

肝内胆管はdetergentsや菌体成分および産生物質などのPAMPに対する固有の防御機構を有しており，生理学的および病的状態で胆管細胞はあらゆる免疫応答を示す．例えば，PBCの胆管上皮にはMHC class IIが異常発現しており，胆管細胞自身が何らかの抗原を提示していると考えられる[58]．また，

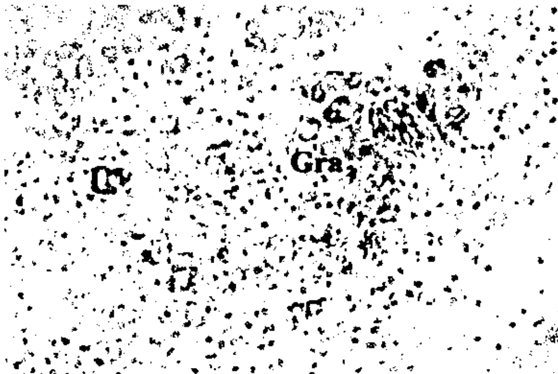

図3 CD1d の免疫組織化学的検出
PBC の肉芽腫（Gra）に CD1d の発現を認める．

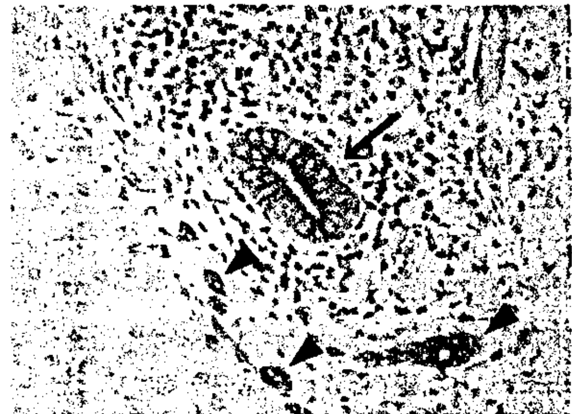

図4 TLR4 の免疫染色
PBC の小葉間胆管（矢印）および細胆管（矢頭）に TLR4 の発現を認め，小葉間胆管は膜状の発現を示す．

PBC の胆管上皮には *in vivo*，*in vitro* に関わらず PDC-E2 の異常発現がみられ[59,60]，PDC-E2 と交差反応する外来性抗原（細菌由来など）の異常集簇であると考えられている[61,62]．PBC の門脈域にはこのような菌体成分に対する免疫応答を示唆する病理学的所見がみられ，胆管細胞と菌体成分との直接的な相互作用も PBC の病態解明に重要な課題である．

1. CD1d と Natural killer-T（NKT）細胞

CD1d は MHC class I 様の抗原提示分子で，糖脂質成分（主に細菌に由来する）を T 細胞に提示する分子群である．PBC の胆管上皮および肉芽腫に CD1d の発現が見られ，何らかの菌体成分が抗原提示されている可能性が示唆される（図 3）[63]．PBC の障害胆管周囲には，燐脂質成分（胆汁に由来）を含む泡沫細胞が多数出現しており，これらの細胞が細菌壁成分である LPS（グラム陰性菌）やリポタイコ酸（グラム陽性菌）などの脂質成分や胆汁中の脂質成分の抗原提示に関与している可能性がある．

NKT 細胞は NK 細胞マーカーと T 細胞受容体を共有するユニークな細胞集団であり，上記 CD1d 分子と結合した糖脂質を認識する細胞である[64]．また，NKT 細胞は IFN-γ や IL-4 などのサイトカインの産生により Th1 や Th2 型 T 細胞への誘導を促し，また Fas-Fas リガンドを介したアポトーシスを誘導する細胞障害性エフェクター細胞としても作用する．この NKT 細胞が PBC の肝組織内，特に障害胆管周囲で増加しており，胆管から CD1d と共に表出されている菌体由来の糖脂質成分を認識し，PBC 特異的な胆管周囲の Th1 型サイトカインネットワークの形成や胆管細胞のアポトーシス誘導に加担している可能性がある[65~68]．

2. スカベンジャー受容体

マクロファージ・スカベンジャー受容体（MSR）は酸化 LDL と結合する受容体であり，脂質代謝産物など生体内の変性産物の処理や，LPS，リポタイコ酸などの糖脂質成分に対する生体防御などさまざまな機能を有する．特にクラス B-1 型の MSR（SRB1）は肝臓で発現しており，血中から胆汁中へのリポ蛋白やコレステロール輸送に重要な役割を果たす．Tsuneyama ら[27,69]は SRB1 抗体を用いた免疫組織化学的検討にて，SRB1 は疾患に関わりなく類洞内皮細胞や Kupffer 細胞，および門脈域に浸潤する単球細胞の一部に発

現が認められ，またPBCの肉芽腫を構成する類上皮細胞にも発現があることを示している．これらのSRB1陽性単核細胞は，肝類洞内や門脈域内に流入したさまざまな生体内変性物質や外来抗原の貪食・処理に重要な役割を果たしていると考えられ，さらにPBCでは肉芽腫形成にも関与していることを示唆する所見である．

3. Monocyte chemotactic protein（MCP）

MCPファミリーは，主にモノサイトから産生される強力なC-C型chemoattractant cytokine（ケモカイン）である．このうちMCP-1は小葉間胆管や増生細胆管に恒常的に発現しており[69,70]，また，胆管細胞はIL-1やTNF-αなどのproinflammatory cytokineの存在下でMCP-1産生亢進を示す[70]．また，MCP-2，MCP-3陽性の分化したマクロファージがPBCの障害胆管周囲や肉芽腫周囲に浸潤している[69]．これらの所見より，PBCの胆管や門脈域に存在する菌体成分に対する反応としてMCP，TNF-α，IL-1などのケモカインやサイトカインが胆管周囲で誘導され，単核球の動員によるPBC特異的な胆管病変形成や肉芽腫形成に加担していると推測される[69]．

4. 胆管細胞の自然免疫機構

細菌などの微生物に対する免疫応答である自然免疫機構は，マクロファージなどの免疫担当細胞のみならず腸管上皮などの上皮細胞も有しており，特にクローン病や潰瘍性大腸炎などの慢性炎症性腸疾患では自然免疫の破綻または異常が病態形成に関与している．われわれは細菌と胆管病変との直接的な関連性を検討するため，細胞膜結合型菌体認識受容体であるToll-like receptor（TLR）の発現について検討した．その結果，胆管細胞はTLR4（LPSの受容体）および関連因子（MD-2）の発現があり，LPS刺激に対して細胞内シグナル伝達分子であるNF-κBが活性化され，TNF-αの産生が誘導されることを報告した[29]．また，免疫組織化学的検討により肝内胆管はTLR4を発現していることを確認しており（図4），生体内でも胆管細胞はLPSなどのPAMPを認識し，胆管固有の自然免疫機構を形成していると考えられる．その他，胆管細胞は2本鎖RNAの受容体であるTLR3も発現しており，ウイルスを直接認識し得る可能性がある．なお，マウスを用いた検討では，上記レトロウイルスの項で記載したmouse mammary tumor virus（MMTV）の認識にはTLR4を使うとの報告もある[71]．PBCの病態形成に胆管系自然免疫機構がどのように関与しているかは現在のところ不明であるが，われわれは微生物に対する過剰な生体反応またはトレランスの破綻がPBCの胆管炎の発生に加担していると想定している．

4 まとめ

PBCの病態発生機序として，ヒトPDC-E2と類似した外来性抗原，特に微生物由来ペプチドとの分子相同性による自己免疫機序が，現在のところ最も有力な仮説であろう．分子生物学的または血清学的検討にていくつかの菌種，ウイルスが候補として報告されているが，特に微生物自体の検出に関しては賛否両論である．考えられる理由として，対象としたPBC患者の病期や肝組織検体の違い（病初期vs肝硬変期，針生検vs移植肝），さらに分子生物学的検出ではプライマー配列の特異性とデザインによるPCR感度の相違に起因するところが大きいようである．また，PBCの病態発生を自然免疫と獲得免疫の2段階の免疫応答の異常（または破綻）で想定

した場合，微生物は初段階時の自然免疫におけるinitiationとして関与するだけで，AMAの出現や胆管破壊などの獲得免疫による病態発生時には微生物の存在を必要としないのかもしれない．PBCのみならずPSCなどの肝胆道系疾患でも病態形成に微生物の関与が十分に考えられ，今後の更なる報告が待たれる．

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Human cord blood- and bone marrow-derived CD34$^+$ cells regenerate gastrointestinal epithelial cells

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SPECIFIC AIMS

In the present study, we aimed to clarify if human cord blood and bone marrow contained progenitor cells that would generate gastrointestinal epithelial cells in clinical and experimental transplantation settings.

PRINCIPAL FINDINGS

1. Three-dimensional analysis excludes the possibility of cell overlay

We analyzed gastrointestinal specimens derived from sex-mismatched female recipients of cord blood (CB), bone marrow (BM), and mobilized peripheral blood (MPB) transplantations to determine whether each hematopoietic cell source contained progenitor cells to generate gastrointestinal epithelial cells. To rule out the possibility of cell overlay using FISH analysis, we performed detailed analysis on signals for a human Y chromosome probe using laser scanning confocal microscopy (**Fig. 1**). Fluorescent and Nomarksy images were obtained from intestinal specimens, which were subjected to FISH analysis using a Spectrum green-conjugated Y chromosome probe and nuclear staining with DAPI (Fig. 1*A–E*). To clarify the localization of signals for chromosomes inside or outside of nuclei, we obtained 12 serial images from different levels at 0.4 μm intervals (Fig. 1*F*). X-Z imaging reconstructed from serial X-Y images demonstrated that the FITC-labeled signal for the human Y chromosome was localized inside the nucleus (Fig. 1*G*).

2. Generation of gastrointestinal epithelial cells in human allogeneic recipients

To further identify the types of donor-derived cells, we performed FISH analysis and immunostaining on the same specimen, not using serial sections of the specimen. We obtained four types of information from each specimen; the nature of nuclei, contour of the cells, antigen expression, and origin of the cells. The FISH and immunofluorescence analyses for different portions of specimens demonstrated that the incidence of donor-derived epithelial cells was between 0.4% and 1.9%. In the present study using specimens derived from pediatric and juvenile recipients without experience of pregnancy, fetal-derived microchimerism can be excluded as a mechanism for the presence of Y chromosome$^+$ cells. The three sources of stem cells, CB-, BM-, and MPB, contained stem/progenitor cells, which could give rise to epithelial cells in allogeneic recipients.

3. Human CB- and BM-derived CD34$^+$ cells give rise to epithelial cells in NOD/SCID/β2M^{null} mice

Next, we examined the capacity of "purified" human progenitor cells in an experimental transplantation setting. We transplanted 1×10^5 human CB- or BM-derived CD34$^+$ progenitor cells into newborn NOD/SCID/β2M^{null} mice, which exhibited extremely low activity of NK cells as well as complete lack of mature B cells and T cells. At 3 months post-transplantation, high levels of engraftment by human CD45$^+$ cells and multilineage reconstitution of human progenitor cells (CD34$^+$), myeloid lineage cells (CD33$^+$), B-lineage cells (CD19$^+$), and T-lineage cells (CD3$^+$) were observed in the xenogeneic host BM. High levels of hematopoietic engraftment by human cells is considered essential to support a relatively low incidence of

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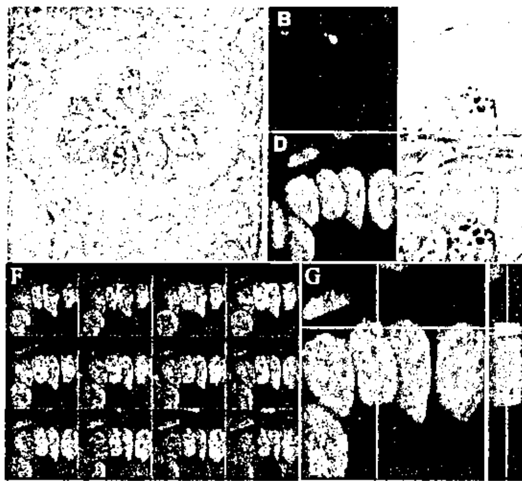

Figure 1. Localization of signal for Y chromosome in the nucleus. In sex-mismatched allogeneic transplantation, donor-derived intestinal epithelial cells were examined with FISH analyses. *A)* Low-magnified image showed that a coronal section of crypt contained a donor-derived cell (arrow) identified with the Spectrum green-conjugated Y chromosome probe. *B)* The existence of the male-derived cell was confirmed with Spectrum green-conjugated Y chromosome probe in a higher magnified view. *C)* Nomarsky imaging revealed the detailed morphology of the cells. *D)* Nuclei of the cells were stained with DAPI. *E)* Images of panels *B–D* merged. *F)* Signals for Y chromosome and nuclei staining are shown in twelve serial images obtained from different levels at 0.4 μm interval. *G)* The location of signal for human X chromosome was determined at the same level of nucleus by X-Z image.

generation of hematopoietic tissue-derived epithelial cells. Similar to the analysis of clinical specimens, the gastrointestinal tissues of recipient mice were analyzed for the presence of human cells by performing FISH analysis and immunofluorescent studies of the same specimens. The incidence of human $CD34^+$ cell-derived epithelial cells in xenogeneic intestinal or gastric tissue was 0.23–0.58% in CB recipients and 0.15–0.30% in BM recipients.

4. The mechanism for the generation of human CB-derived intestinal epithelial cells

We examined the possibility of cell fusion as a mechanism for the generation of CB- or BM-derived epithelial cells. When we examined the presence of human cells in mesenteric lymphoid nodes, the vast majority of cells were dually positive for human X chromosome and human CD45 (**Fig. 2*A***), showing that human $CD34^+$ progenitor cells could effectively reconstitute lymphoid tissues of the recipient intestine. As no $cytokeratin^+$ epithelial cells were stained with anti-human CD45 antibody, we performed double FISH analyses using human and murine chromosome probes and immunostaining for cytokeratin on the same specimen. Nomarsky imaging demonstrated that a human CB-derived human X $chromosome^+$ cell was identified in organized sequence of epithelial cells in villi (Fig. 2*B*). In Fig. 2*C*, a human X $chromosome^+$ cell was positively stained with anti-cytokeratin antibody, suggesting that the human cell was an epithelial cell. We tested whether human X $chromosome^+$ epithelial cells with

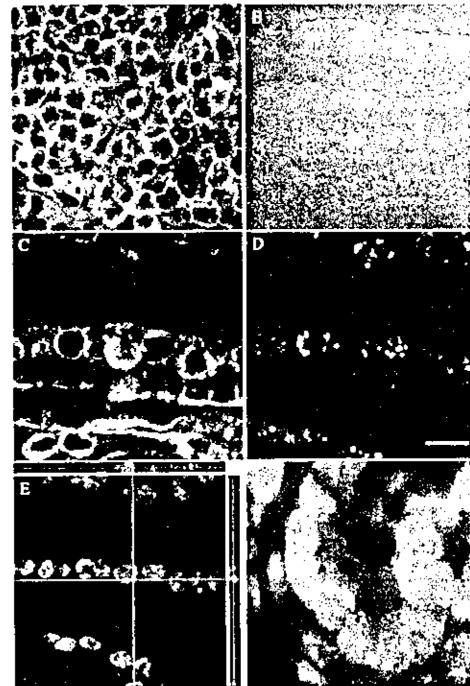

Figure 2. Human X chromosome-positive epithelial cell was not generated by the fusion between human cell and murine epithelial cell. Human CB-derived $CD34^+$ cells were transplanted into newborn NOD/SCID/$\beta 2M^{null}$ recipients. At 3 months post-transplantation, in vivo generation of human intestinal epithelial and lymphoid cells was examined by laser confocal microscopy. *A)* Lymphoid tissue derived from a recipient intestine was subjected to immunostaining with anti-human CD45 antibody (green) and FISH analysis with human X chromosome probe (red). The majority of the cells in lymphoid tissue of the recipient mice were human hematopoietic cells as judged by the positivity of human X chromosome and human CD45. *B–E)* After FISH analyses and immunofluorescent studies were performed on the same specimen derived from recipient intestine, four-color (FITC, Spectrum orange, Cy5, DAPI) analyses were executed with laser scanning confocal microscopy along with Nomarsky imaging. *B)* Nomarsky image showed that human cell identified with anti-human X chromosome probe (red) was identified in organized sequence of villi. *C)* The cell with human X chromosome was confirmed as an epithelial cell by positive staining with Cy5-conjugated anti-cytokeratin antibody. *D)* The majority of the cells in the same specimen were murine cells, which were identified with FITC-conjugated mouse pan-centromeric probe. The cell identified with human X chromosome probe did not possess any mouse centromeres in the nucleus. Bar represents 10 μm. *E)* The hybridization signal for human X chromosome was located inside nucleus as judged by X-Z image reconstructed from serial X-Y images. (F) Such human cells were also identified at the bottom of crypt.

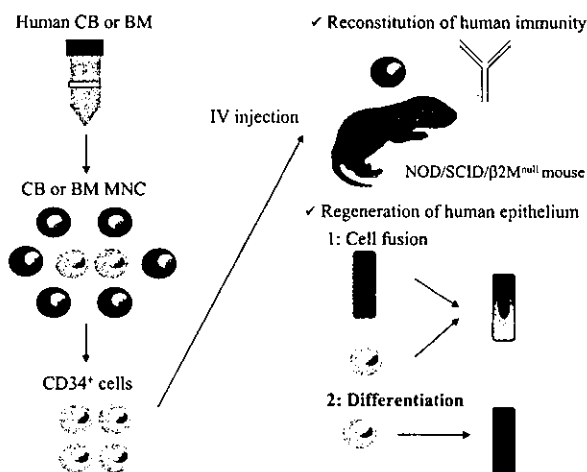

Figure 3. Schematic diagram.

donor chromosomes were generated due to the fusion of donor-derived progenitors and recipient-derived epithelial cells. Although the majority of epithelial cells had murine centromeres, simultaneous FISH analyses using species-specific probes demonstrated that the nucleus of the human X chromosome$^+$ cell was not labeled with the mouse centromere probe (Fig. 2*D*), indicating that the human chromosome$^+$ epithelial cell was not generated by fusion of human CD34$^+$ cells and murine epithelial cells. X-Z imaging reconstructed from 10 slices of 0.3 μm serial X-Y images confirmed that the dot indicating the presence of human X chromosome was located inside the nucleus of the epithelial cell (Fig. 2*E*) and not was an artifact due to cell overlay. Such human X chromosome$^+$ cells were identified both along villi and at the bottom of crypt (Fig. 2*F*), a candidate location of intestinal stem cells.

CONCLUSIONS AND SIGNIFICANCE

We have used clinical and experimental transplantation settings in order to examine the capacity of human hematopoietic progenitor cells to generate gastrointestinal epithelial cells in allogeneic and xenogeneic recipients.

In terms of technical issue, we successfully combined FISH analysis with immunofluorescent studies to identify donor chromosome$^+$ cytokeratin$^+$ cells. The simultaneous FISH and immunofluorescent studies of the same specimens, not using serial sections, enabled the identification of donor-derived epithelial cells accurately. One of the criticisms against previous FISH studies was that a tiny dot indicating the presence of donor chromosome could be localized at a different level of the nucleus due to cell overlay. Both in allogeneic and xenogeneic transplantations, X-Z images, reconstructed from serial X-Y images captured by laser scanning confocal microscopy, clearly demonstrated that the hybridization signal for human chromosome existed inside the nucleus, which supported the specificity of FISH analyses in the present study. Our analyses of pediatric and juvenile recipients demonstrated that Y chromosome$^+$ epithelial cells of gastrointestinal tract were derived from transplanted hematopoietic tissue-derived cells, not reminiscent of fetus-derived cells.

Considering the limited availability of clinical specimens and the controversy regarding the generation of BM-derived epithelial cells in clinical cases, we developed experimental xenotransplantation assays, in which purified human progenitor cells could be analyzed for their capacity. We here identified that human CB- and BM-derived CD34$^+$ cells were capable of generating epithelial cells in vivo. Although the capacities of CB and BM did not differ significantly in the present study, CB could be used as precious source for allogeneic and autologous progenitor cells as well as BM in the future regenerative medicine.

Molecular mechanism underlying stem cell plasticity has yet to be understood. Cell fusion between donor-derived stem cells and mature cells of recipient origin may account for seemingly donor stem cell-derived progeny. As hepatocytes and myocytes are known to fuse in physiological condition, the mechanism for the generation of CB- and BM-derived gastrointestinal epithelial cells needs to be clarified. Sex chromosome painting in the FISH analysis of clinical specimens cannot determine the possibility of cell fusion as thin sections do not include the whole chromosomes. Xenogeneic assay enabled us to evaluate the possibility of cell fusion by using human chromosome probe and mouse centromere probe on the same specimens. Double FISH analyses demonstrated that the cells positive for human chromosome and cytokeratin were not labeled with anti-mouse centromere probe. Altogether, it is concluded that fusion between stem cells and epithelial cells is not the only mechanism at least in intestine and that human purified progenitor cells can regenerate intestinal epithelial cells across allogeneic and xenogeneic histocompatibility barrier. FJ

Tyrosine Kinase 2 Interacts with and Phosphorylates Receptor for Activated C Kinase-1, a WD Motif-Containing Protein[1]

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Receptor for activated C kinase (Rack)-1 is a protein kinase C-interacting protein, and contains a WD repeat but has no enzymatic activity. In addition to protein kinase C, Rack-1 also binds to Src, phospholipase Cγ, and *ras*-GTPase-activating proteins. Thus, Rack-1 is thought to function as a scaffold protein that recruits specific signaling elements. In a cytokine signaling cascade, Rack-1 has been reported to interact with the IFN-αβ receptor and Stat1. In addition, we show here that Rack-1 associates with a member of Jak, tyrosine kinase 2 (Tyk2). Rack-1 interacts weakly with the kinase domain and interacts strongly with the pseudokinase domain of Tyk2. Rack-1 associates with Tyk2 via two regions, one in the N terminus and one in the middle portion (aa 138–203) of Rack-1. Jak activation causes the phosphorylation of tyrosine 194 on Rack-1. After phosphorylation, Rack-1 is translocated toward the perinuclear region. In addition to functioning as a scaffolding protein, these results raise the possibility that Rack-1 functions as a signaling molecule in cytokine signaling cascades. *The Journal of Immunology*, **2004, 173: 1151–1157.**

Many cytokines bind to specific cell surface receptors and activate members of the Janus family of protein tyrosine kinases (Jaks), which are associated with cytokine receptors (1). The activated Jaks phosphorylate the tyrosine residues of the receptors, thereby recruiting Stats and other signaling molecules into the activated receptor complex. Stats are then phosphorylated by Jaks, and are subsequently translocated to the nucleus, where they affect gene expression. This Jak-Stat signaling pathway is widely used by members of the cytokine receptor super family (2). There are four mammalian Jaks: Jak1, Jak2, Jak3, and tyrosine kinase 2 (Tyk2).[3] Tyk2 has been identified as a novel protein kinase which can compensate for IFN-α nonresponsive mutated fibroblasts (3). IFN-α specifically activates Jak1 and Tyk2, which phosphorylate Stat 1 and 2. These activated Stats subsequently associate to form either Stat1 homodimers or the transcription factor ISGF-3, which then translocate to the nucleus to regulate gene expression (2, 4). Both Tyk2 and Jak1 were thought to be essential for signal transduction downstream of IFN-α in mutated fibroblasts, which are not responsive to IFN-α (4). However, using Tyk2-deficient mice, we and others have shown that Tyk2 has a restricted function and does not play a major role in IFN-α signaling (5, 6). In contrast, Jak 1-null cells fail to respond to IFN-α (7). In addition, Stat1-null mice are defective in almost all IFN-α-induced responses (8, 9). Stat2-null mice also demonstrated an increased susceptibility to viral infection (10). Based on these data, the Jak1-Stat signaling pathway is thought to be essential for IFN-α signaling. Recently, we reported that Tyk2 was essential for IFN-α-induced B lymphocyte growth inhibition (11). Stat1 is not required for this IFN-α-mediated inhibition (12); therefore, other signaling molecules must exist downstream of activated Tyk2 to transduce the IFN-α signal inhibiting B lymphocyte growth. Thus, we performed a yeast two-hybrid screen for proteins that interact with Tyk2.

In this report, we identify receptor for activated C kinase (Rack)-1, originally described as a receptor for activated C kinase β, as a protein that interacts with Tyk2. Rack-1 has previously been reported to constitutively interact with the β long subunit of the type I IFNR (13), Jak1, Tyk2 (14), and Stat1 (15). We show here that Rack-1 associates with Jaks, and is phosphorylated on tyrosine residues by Jaks. This raises the possibility that Rack-1 functions as a signaling molecule in cytokine signaling cascades.

Materials and Methods

Abs and reagents

Anti-Rack-1, -Tyk2, -Jak1, -Jak2, and -Jak3 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine mAb (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-c-Myc mAb was purchased from BD Clontech (Palo Alto, CA). Anti-Flag M2 mAb was purchased from Sigma-Aldrich (St. Louis, MO). Murine IFN-α was purchased from HyCult Biotechnology (Uden, The Netherlands).

Yeast two-hybrid screen

A cDNA encoding the kinase domain (aa 833-1187) of human Tyk2 was subcloned into the Gal DNA-binding domain plasmid pGBKT7 (BD Clontech), and was used as bait in a yeast two-hybrid screen of a human B lymphocyte cDNA library constructed in pACT (BD Clontech). Approximately 1.6×10^6 colonies were screened for activation of the ADE2, HIS3, and *lacZ* reporter genes using the host strain AH109. The inserts from positive library plasmids were then amplified by PCR and mapped by *Alu*I digestion. Plasmids were sequenced after isolation and bacterial transformation.

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[3] Abbreviations used in this paper: Tyk2, tyrosine kinase 2; Rack, receptor for activated C kinase; PKC, protein kinase C.

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Mammalian expression vector constructs

Murine Jak1, Jak2, Jak3, and human Tyk2 expression constructs were gifts from Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). Kinase-negative versions of the Jak proteins were generated by mutating lysine (K833) to glutamine in murine Jak1 (Jak1 KE) and lysine (K882) to glutamine in murine Jak2 (Jak2 KE) (16).

The partial human Tyk2 cDNAs expressing the various domains (aa 1–450, 266–733, 600–1086, or 833–1187) indicated in Fig. 3*A* were generated by PCR, and were subcloned into pCMV-MyC (BD Clontech). The clone 4-86 cDNA (C-terminal region of Rack-1 encoding aa 137–317), whose gene product associated with Tyk2 (aa 833–1187) in yeast, was removed from pACT2 by *Sfi*I/*Xho*I digest, and was subcloned into pCMV-HA (BD Clontech). The full-length human Rack-1 cDNA was generated by RT-PCR from human peripheral blood lymphocytes, and was subcloned into pCR2.1-TOPO (Invitrogen Life Technologies, Carlsbad, CA). *Hind*III-*Apa*I, *Xho*I, *Bam*HI-*Eco*RI, *Bgl*II-*Eco*RI, and *Bam*HI fragments of the full-length Rack-1 were subcloned into the Flag-tagged mammalian expression plasmid pCMV-Tag2 (BD Clontech) to generate Rack-1 (aa 1–317), (aa125–317), (aa 204–317), (aa 258–317), and (aa 1–204) indicated in Fig. 3C. Rack-1 (1–137) was generated by PCR using Rack-1 in pCR2.1-TOPO as the template, and was subcloned into the *Bam*HI site in pCMV-Tag2. Rack-1 (Δ138–203) was generated by ligation of the aa 1–137 fragment to the 204–317 fragment in pCMV-Tag2.

Oligonucleotide-directed mutagenesis was used to substitute phenylalanine for tyrosine at residues 140, 194, 228, and 246 of wild-type Rack1 in pCMV-Tag2. The Transformer site-directed mutagenesis kit was used according to the manufacturer's protocol (BD Clontech) with the following oligonucleotides: Y140F oligo, GGTGTGTGCAAATTCACTGTCCAG; Y194F oligo, CACACAGGCTTTCTGAACACGGTG; Y228F oligo, GGCAAAC ACCTTTTCACGCTAGAT; Y246F oligo, CCTAACCGCTTCTGGCTGT GTGCT.

Cell culture and transfection

HEK293T cells were plated at 2–4 × 10^6 cells/ml in DMEM (Sigma-Aldrich) containing 10% heat-inactivated FBS (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine (Invitrogen Life Technologies, Gaithersburg, MD), MEM nonessential amino acids (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), 100 U/ml penicillin, and 10 μg/ml streptomycin (Invitrogen Life Technologies), and grown at 37°C in 5% CO_2 to 60% confluence. 293T cells were transfected with 10 μg of plasmid DNA using the calcium phosphate precipitation protocol. Cells were cultured to almost 100% confluence and harvested, after appropriate stimulation.

The IL-3-dependent cell line, BAF/3, was maintained in RPMI 1640 medium supplemented with 10% FCS and IL-3.

Immunoprecipitation and immunoblotting

Cells were lysed as previously described (16). Cell lysates were centrifuged at 12,000 × *g* for 15 min to remove debris. For immunoprecipitation, the indicated Abs were added to the supernatant of each sample, incubated for 8 h, and mixed with protein A-agarose (Sigma-Aldrich). Total cell lysates or immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were probed using the appropriate Abs and visualized by ECL (Amersham Biosciences).

Immunofluorescence

HEK293T cells were maintained in DMEM containing 10% FCS and transfected with Flag-tagged wild-type or E mutant Rack-1 with or without Tyk2, Jak1, Jak2, the Jak1 KE mutant, or the Jak2 KE mutant by the calcium phosphate precipitation protocol. Forty-eight hours after transfection, cells were fixed with a solution containing 4% paraformaldehyde and incubated with an anti-Flag Ab. The cells were then incubated with a FITC-conjugated anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) and mounted with a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were observed using a confocal laser fluorescence microscope. The intracellular localization of labeled Rack-1 was assessed in reference to nuclear 4′6′-diamidine-2-phenylindole dihydrochloride (DAPI) staining. The digital images were processed by layering and the contrast of all images was increased by 50% using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA). Nuclear DAPI staining appears blue and Rack-1 appears green.

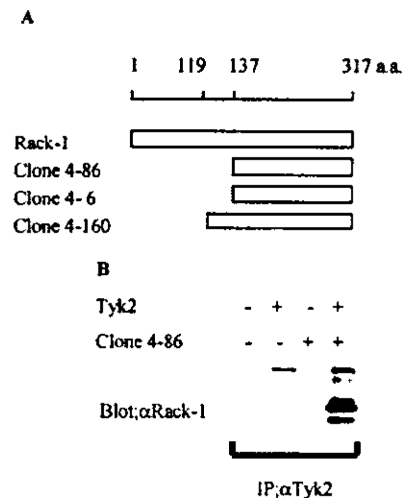

FIGURE 1. Rack-1 interacts with Tyk2. *A*, A schematic representation of the Rack-1 protein identified by yeast two-hybrid screen using the Tyk2 tyrosine kinase domain as bait. Amino acids 137–317 of Rack-1 were present in three clones which interacted with Tyk2 in yeast cells. *B*, Coimmunoprecipitation of clone 4-86 with Tyk2 in mammalian 293T cells. 293T cells were transfected with clone 4-86 and/or Tyk2. Cell lysates were immunoprecipitated with anti-Tyk2 Ab, and immunoblotted with anti-Rack-1 Ab.

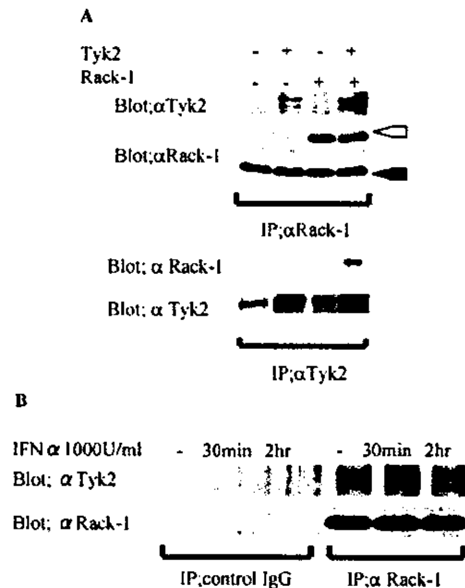

FIGURE 2. The physical interaction between Rack-1 and Tyk2 is independent of IFN stimulation. *A*, 293T cells were transfected with Tyk2 and/or Rack-1. *Top*, Cell lysates were immunoprecipitated with anti-Rack-1 Ab and immunoblotted with anti-Tyk2 (*upper panel*) or anti-Rack-1 Ab (*lower panel*). Open arrow indicates transfected Rack-1, and the filled arrow indicates endogenous Rack-1. *Bottom*, Cell lysates were immunoprecipitated with anti-Tyk2 Ab and immunoblotted with anti-Rack-1 Ab (*upper panel*) or anti-Tyk2 Ab (*lower panel*). *B*, BAF/3 cells were either stimulated for the indicated time with IFN-α (1000 U/ml) or left unstimulated as a control. Cell lysates were immunoprecipitated with nonspecific mouse IgG or anti-Rack-1 Ab and immunoblotted with anti-Tyk2 Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*).

Results

Identification of Rack-1 as a Tyk2-interacting protein

To identify novel Tyk2-interacting proteins, a yeast two-hybrid screen was performed using the kinase domain of Tyk2 fused to the Gal DNA-binding domain as bait. A human bone marrow cell cDNA library was screened. Among a number of positive clones, three clones termed 4-6, 4-86, and 4-160, encoded the C-terminal portion of the previously described Rack-1 (Fig. 1*A*). To determine whether these clones encoded a protein able to interact with Tyk2 in mammalian cells, clone 4-86 was subcloned into the mammalian expression vector pCMV-HA. The resulting expression vector produced a protein that interacted strongly with Tyk2 in 293T cells (Fig. 1*B*), suggesting that the region comprising aa 137–317 of Rack-1 contains the binding site for Tyk2.

We next examined the association between Tyk2 and Rack-1 using the full-length cDNA in mammalian cells. The full-length Flag tagged Rack-1 cDNA was transiently transfected into 293T cells with or without the full-length Tyk2 expression vector. Fig. 2*A* shows that Tyk2 coimmunoprecipitated with Rack-1 and Rack-1 coimmunoprecipitated with Tyk2 when both Tyk2 and Rack-1 were expressed in 293T cells. We next investigated whether endogenous Rack-1 was able to interact with Tyk2 and how this might be affected by the activation of Tyk2. To activate Tyk2, a mouse pro-B cell line, BAF/3, was stimulated or not stimulated with IFN-α. Tyk2 immunoprecipitates were assessed for the presence of endogenous Rack-1 by Western blotting. As can be seen in Fig. 2*B*, Tyk2 was present in the Rack-1 immunoprecipitates. Furthermore, equivalent amounts of Tyk2 were present in samples which had or had not been stimulated with IFN-α. This indicates that Rack-1 associates with Tyk2 in BAF/3 cells, and that this interaction is not altered by stimulation with IFN-α.

Mapping the sites in Tyk2 and Rack-1 required for binding

We next sought to determine which region of Tyk2 was responsible for the interaction with Rack-1. We separated the Tyk2 cDNA into four regions (Fig. 3*A*), and each region was subcloned in frame with a *myc* tag in the mammalian expression vector pCMV-Myc. The full-length Tyk2 construct or each partial Tyk2 construct was transiently transfected into 293T cells along with the full-length Rack-1 construct. Cell lysates were immunoprecipitated with anti-Tyk2 or anti-Myc Ab, and Western blotted with

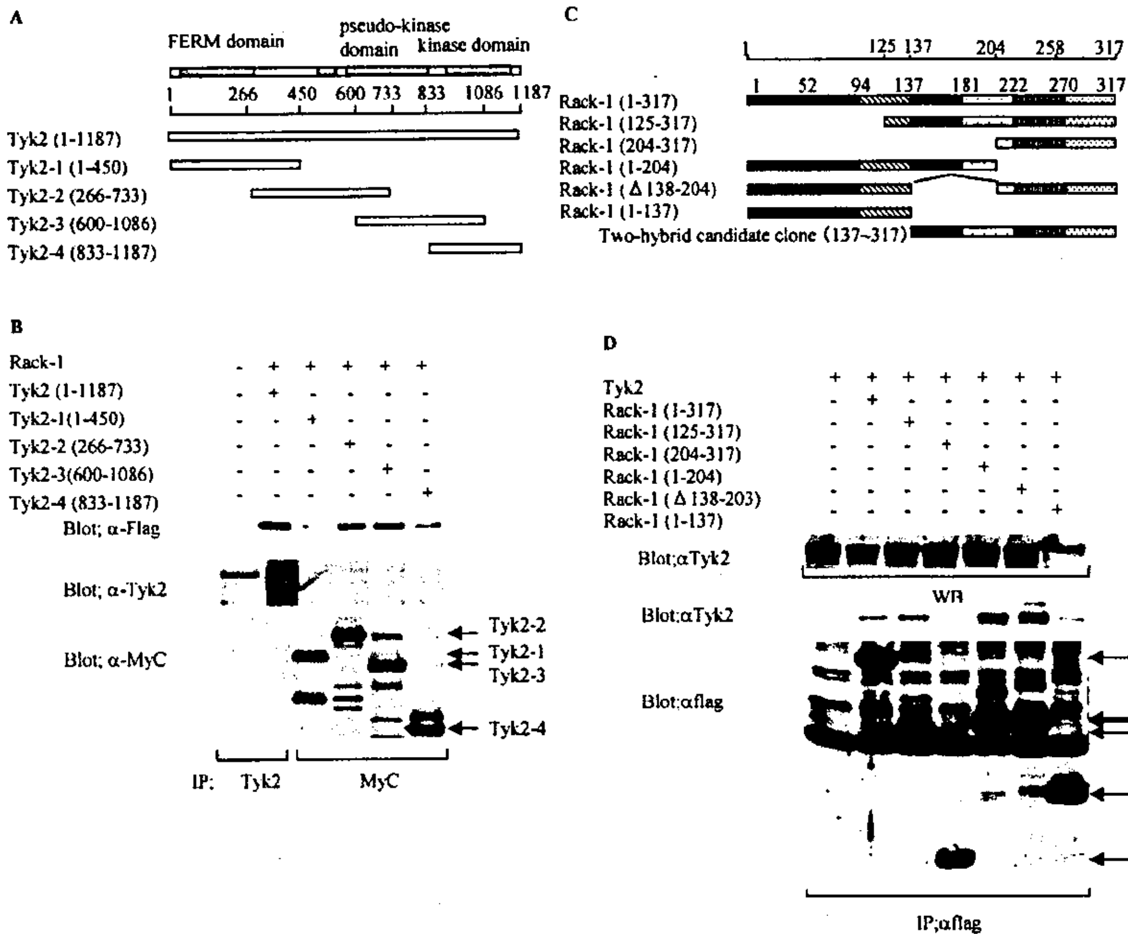

FIGURE 3. Mapping the sites in Tyk2 and Rack-1 required for binding. *A*. A schematic of the domain structure of Tyk2 and the mutant fragments. *B*. Full-length Tyk2 or Myc-tagged Tyk2 mutants were coexpressed with Flag-tagged Rack-1 in 293T cells. Forty-eight hours after transfection, the cells were lysed and immunoprecipitations were performed with anti-Tyk2 or anti-Myc Ab. The immunoprecipitates were immunoblotted with anti-Flag Ab (*upper panel*), anti-Tyk2 Ab (*middle panel*), or anti-Myc Ab (*lower panel*). *C*, A schematic of the domain structure of Rack-1 and the mutant fragments. *D*, Rack-1 mutants were coexpressed with Tyk2 in 293T cells. Forty-eight hours after transfection, the cells were lysed and immunoprecipitations were performed with anti-Tyk2 Ab. The immunoprecipitates were immunoblotted with anti-Flag Ab (*upper panel*) or anti-Tyk2 Ab (*lower panel*).

anti-flag Ab. Fig. 3*B* shows that Rack-1 interacted with Tyk2 (266–733) and Tyk2 (600–1086) as well as full-length Tyk2. In addition, Rack-1 interacted weakly with Tyk2 (833–1187), which contains the kinase domain of Tyk2. As Rack-1 did not interact with Tyk2 (1–450), these data indicate that Rack-1 interacts weakly with the kinase domain of Tyk2 and interacts strongly with the pseudokinase domain of Tyk2. Furthermore, as there is no overlapping region in Tyk2 (266–733) and Tyk2 (833–1187), there is the possibility that multiple RACK-1 binding sites exist in Tyk2.

We next determined the region of Rack-1 required for the interaction with Tyk2. Fig. 3*C* shows the full-length and deletion mutants of Rack-1 used in this experiment. As shown in Fig. 3*D*, full-length Rack-1 (1–317), Rack-1 (125–317), and Rack-1 (1–204) bound to Tyk2. The C-terminal region of Rack-1 (aa 204–317) did not associate with Tyk2. Taken together with the fact that the two-hybrid candidate clone 4-86 encodes aa 137–317 of Rack-1, these data suggest that the middle portion of Rack-1, aa 137–203, may associate with Tyk2. We next examined the binding of Rack-1 Δ 138–203, which lacks aa 138–203, to Tyk2. Unexpectedly, Rack-1 Δ 138–203 also binds to Tyk2 (Fig. 3*D*). This indicates that two regions of Rack-1, the N terminus and the middle portion, might bind to Tyk2. This was confirmed by the fact that Rack-1 (1–137) binds to Tyk2 (Fig. 3*D*).

Rack-1 is phosphorylated on Tyr^{194}, a residue in the fifth WD repeat, by Jaks

As Tyk2 is a tyrosine kinase, we assessed whether Rack-1 could be phosphorylated by Tyk2. When Rack-1 was transiently cotransfected into 293T cells with Tyk2, Rack-1 was phosphorylated by Tyk2 (Fig. 4*A*). There are six tyrosine residues in Rack-1; to identify the specific tyrosine residue(s) that is phosphorylated by Tyk2 in vivo, we performed site-directed mutagenesis, substituting phenylalanine for tyrosine at individual and multiple sites in Rack-1 (Fig. 4*B*). The Rack-1 mutants were then transiently coexpressed with Tyk2 in 293T cells, and proteins were immunoprecipitated with anti-Rack-1 and subjected to immunoblot analysis with anti-phosphotyrosine (Fig. 4*C*). We found that the Rack-1 mutants containing phenylalanine at position 194 (the E, F, H, and I mutants) were not phosphorylated by Tyk2. Because the Rack-1 E mutant is a single substitution of tyrosine 194 to phenylalanine and this mutant is not phosphorylated, Tyk2 must phosphorylate Rack-1 on Tyr^{194}. In addition, the immunoprecipitates of all Rack-1 mutants contained phosphorylated Tyk2, indicating that the tyrosine phosphorylation of Rack-1 has no influence on the association of Rack-1 and Tyk2. This result was confirmed by the transient transfection of wild type or the E mutant of Rack-1 with or without Tyk2 in 293T cells. Proteins were immunoprecipitated with Tyk2 or Rack-1. The results shown in Fig. 5 demonstrate that Rack-1 was present in the Tyk2 immunoprecipitates at equivalent levels whether aa 194 of Rack-1 was tyrosine or phenylalanine. In addition, Tyk2 was present in the Rack-1 immunoprecipitates at equivalent amounts. Taken together, these results demonstrate that phosphorylation of tyrosine 194 of Rack-1 is not important for the interaction of Rack-1 and Tyk2.

We next examined whether other Jaks could phosphorylate Rack-1. In addition to Tyk2, Jak1, Jak2, and Jak3 were able to phosphorylate Rack-1 (Fig. 6). We also transfected kinase-dead Jak1 or Jak2 (KE mutant) with Rack-1 into 293T cells (Fig. 6, *lane 3* and *5*). In this case, Rack-1 was not phosphorylated, although Rack-1 associated with Jak1KE and Jak2KE. This indicates that Jaks directly phosphorylate Rack-1. Furthermore, none of the Jak family kinases phosphorylated the Rack-1 E mutant (Fig. 6, *right*), indicating that all Jaks phosphorylate Rack-1 on a single tyrosine, residue 194. Notably, immunoprecipitates containing the Rack-1 E

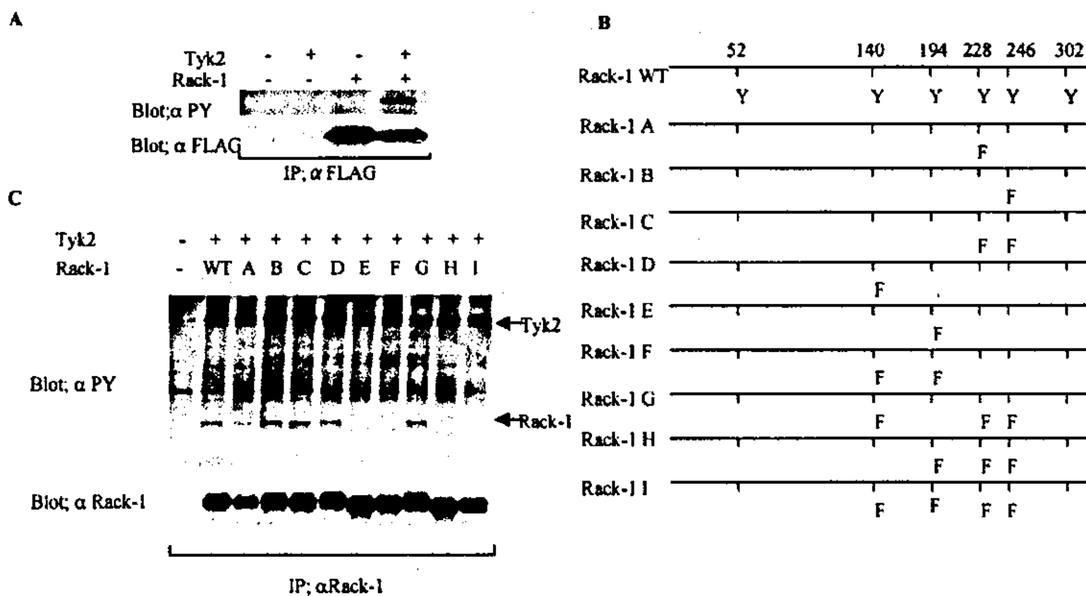

FIGURE 4. Tyk2 phosphorylates Rack-1 on Tyr^{194}, a residue in the fifth WD repeat. *A*, Rack-1 is phosphorylated by Tyk2. Tyk2 and/or Flag-tagged Rack-1 were expressed in 293T cells. Total cell lysates were immunoprecipitated with anti-Flag Ab and immunoblotted with anti-phospho tyrosine Ab (*upper panel*) or anti-Flag Ab (*lower panel*). *B*, Site-directed mutagenesis of Rack-1 was perfomed to substitute phenylalanine for tyrosine at individual and multiple sites. *C*, Tyk2 phosphorylates Rack-1 on Tyr^{194}, a residue in the fifth WD repeat. 293T cells were transfected with a series of wild-type or Rack-1 mutants. Forty-eight hours after transfection, cells were lysed, immunoprecipitated with anti-Rack-1 Ab, and immunoblotted with anti-phosphotyrosine Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*). The arrows indicate tyrosine phosphorylated Rack-1 and Tyk2 which coimmunoprecipitates with Rack-1.

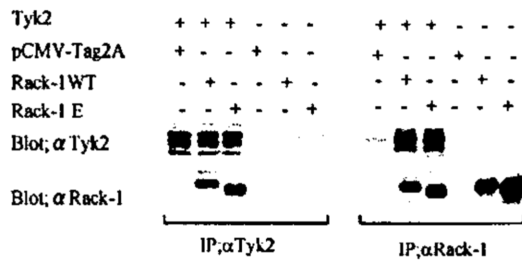

FIGURE 5. Tyrosine phosphorylation of Rack-1 has no influence on the association between Rack-1 and Tyk2. Empty vector (pCMV-Tag2A), wild-type Rack-1, or the Rack-1 E mutant (Y194F) were coexpressed with Tyk2 in 293T cells. Total cell lysates were immunoprecipitated with anti-Tyk2 Ab (*left panel*) or anti-Rack-1 Ab (*right panel*) and immunoblotted with anti-Tyk2 Ab (*upper panel*) or anti-Rack-1 Ab (*lower panel*).

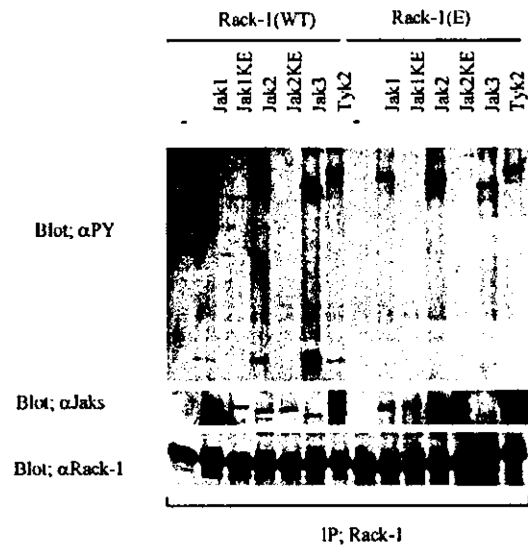

FIGURE 6. In addition to Tyk2, Jak1, Jak2, and Jak3 phosphorylate tyrosine 194 on Rack-1. Wild-type or the Rack-1 E mutant (Y194F) were coexpressed with either Jak1, the Jak1 KE mutant, Jak2, the Jak2 KE mutant, Jak3, or Tyk2 in 293T cells. Total cell lysates were immunoprecipitated with anti-Rack-1 Ab and immunoblotted with anti-phosphotyrosine Ab, anti-Rack-1 Ab, or anti-Jak Abs (mixture or individual anti-Jak1, anti-Jak2, and anti-Tyk2 Abs) as indicated.

mutant contained Jak1, Jak2, Jak3, and Tyk2. Therefore, Jaks associate with Rack-1 whether Rack-1 is tyrosine phosphorylated or not.

Perinuclear translocation of Rack-1 by Jaks

293T cells were used to localize the distribution of Rack-1 within cells with or without the activation of Jaks (Fig. 7*A*). When Rack-1 was transfected into 293T cells, it was detected throughout the cytoplasm. When both Rack-1 and Tyk2 were transfected into 293T cells, intracellular redistribution of Rack-1 toward the perinuclear area was observed. The transfection of Jak1 or Jak2 with Rack-1 in 293T cells also induced the perinuclear translocation of Rack-1. To determine whether the perinuclear translocation of Rack-1 was induced by Jaks, we transfected the Jak1 KE mutant or the Jak2 KE mutant with Rack-1 in 293T cells. Under these conditions, Rack-1 was detected throughout the cytoplasm, and was not translocated to the perinuclear region. Next, we examined whether perinuclear translocation of Rack-1 required the phosphorylation of Rack-1. When the Rack-1 E mutant (Y194F) was transfected with Tyk2 into 293T cells, perinuclear translocation of the Rack-1 E mutant was observed to the same extent as Rack-1 wild type (Fig. 7*B*).

Discussion

The main substrates of Jaks are the Stats, and the phosphorylation of Stats is essential for the biological activities of cytokines (2). Analysis of Stat-deficient mice has demonstrated that Stats transduce almost all cytokine signaling (17). In the case of inhibition of IL-7-induced B cell growth by IFN-α, Stat1 activation was not required (12), although we found that Tyk2 activation was necessary (11). To find Tyk2-activated signaling molecules other than the Stats, we performed a yeast two-hybrid screen for proteins that associated with Tyk2. We found that Rack-1, which was originally described as a receptor for activated protein kinase C (PKC)-β (18), associated with Tyk2 (Fig. 1). Interestingly, it has recently been reported that Rack-1 associates with the β-chain of the type I IFNR and recruits Stat1 to the receptor complex. This raises the possibility that Rack-1 serves as a scaffolding protein during cytokine signal transduction. We have shown here that Tyk2 associates with Rack-1 in 293 T cells and BAF/3 cells (Fig. 2). This association was not altered by stimulation with IFN-α (Fig. 2*B*). Recently, Usacheva et al. (14) reported that Tyk2 and Jak1 associated with Rack-1 using GST-fusion proteins. Consistent with our result, they also reported that association of Jak1 and Rack-1 was not affected by IFN-β treatment. Additionally, we have determined the binding sites on Tyk2 and Rack-1. Tyk2 binds strongly to Rack-1 through the kinase domain, and binds weakly through the pseudokinase domain (Fig. 3*B*). Therefore, Tyk2 probably associates with Rack-1 through more than one binding site. Similarly, Rack-1 binds to Tyk2 through two regions, one in the N terminus and one in the middle portion of the protein (aa 137–203) (Fig. 3*D*).

As Tyk2 is a tyrosine kinase, we next analyzed whether Rack-1 could serve as a substrate of Tyk2. When Tyk2 and Rack-1 were transiently transfected into 293T cells, Rack-1 was phosphorylated by Tyk2 (Fig. 4*A*). In addition to Tyk2, other members of the Jak family (Jak1, Jak2, and Jak3) phosphorylated Rack-1 (Fig. 6). Kinase-dead Jaks (Jak1 KE or Jak2 KE mutants) were unable to phosphorylate Rack-1, suggesting that Jaks directly phosphorylate Rack-1.

When cells are stimulated by PMA, PKC is activated and Src phosphorylates Rack-1 on Tyr^{228} and/or Tyr^{246} (19). Therefore, we wanted to determine whether the site of Tyk2 phosphorylation was the same as the site of Src phosphorylation. We demonstrated that the A (Y-228-F), B (Y-246-F), and C (Y-228, 246-F) mutants of Rack-1 were phosphorylated by Tyk2 (Fig. 4*C*). Next, we substituted tyrosine 140 and/or 194 to phenylalanine. The substitution of tyrosine 194 to phenylalanine in Rack-1 (this mutation is present in the E, F, H, and I mutants) diminished the phosphorylation of Rack-1 by Tyk2 (Fig. 4*C*). In addition to Tyk2, other members of the Jak family (Jak1, Jak2, and Jak3) phosphorylated tyrosine 194 on Rack-1 (Fig. 6). Therefore, Jaks phosphorylate only tyrosine 194 of Rack-1. In addition, these results indicate that Jaks and Src kinase phosphorylate different tyrosine residues on Rack-1.

Although the binding of Rack-1 to Src required the phosphorylation of Rack-1 (19), Rack-1 mutants (E, F, H, and I) which were not phosphorylated by Tyk2 were still able to associate with Tyk2 (Fig. 4*C*). Indeed, wild-type Rack-1 and the Rack-1 E mutant associated with Tyk2 to the same degree (Fig. 5). These results indicate that the association of Rack-1 and Tyk2 occurred regardless of Rack-1 phosphorylation, and this association is not enhanced by tyrosine phosphorylation of Rack-1. In addition, catalytic activity

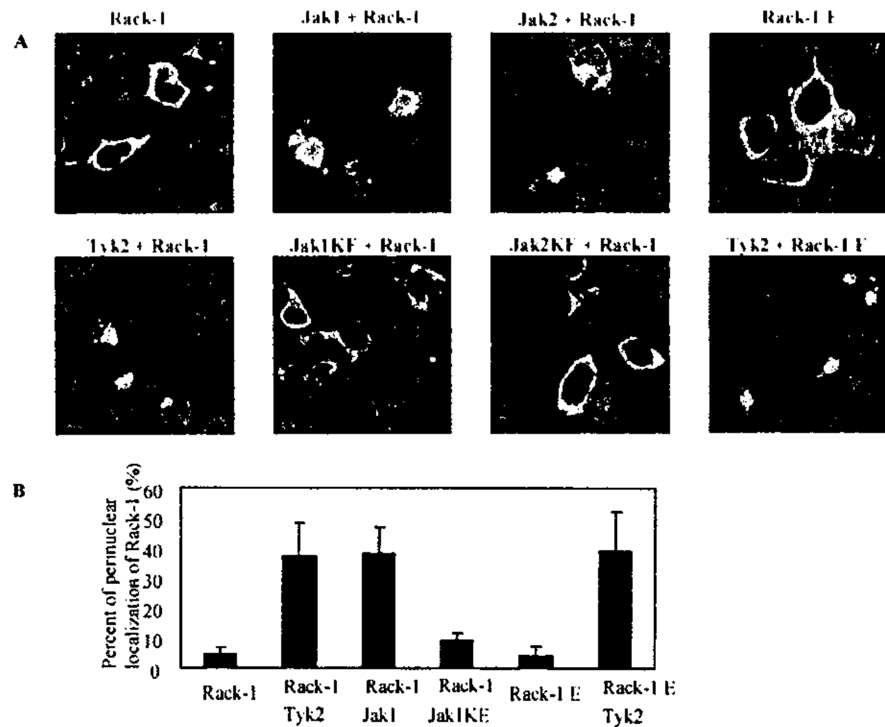

FIGURE 7. Localization of Rack-1. *A*, Flag-tagged Rack-1 (wild type or E mutant) was expressed either alone or in combination with one of Tyk2, Jak1, the Jak1 KE mutant, Jak2, or the Jak2 KE mutant in 293T cells. After 48 h, the localization of tagged proteins was visualized by confocal microscopy. *B*, The number of cells showing perinuclear localization of Rack-1 was calculated. Data represent the mean with SD of four different experiments.

of Jaks is not essential for binding to Rack-1, as the KE mutants of both Jak1 and Jak2 associated with Rack-1 (Fig. 6). This result is consistent with previous observations that Rack-1 associated with Tyk2 in BAF/3 cells in the presence or absence of IFN-α stimulation (Fig. 2*B*).

IFN-β stimulation has been reported to translocate Rack-1 toward the perinuclear region (13). Transient expression of Jaks in 293T cells led to the autophosphorylation of the Jaks. When Rack-1 alone was transfected into 293T cells, Rack-1 was present throughout the cytoplasm. In contrast, when both Jak (Jak1, Jak2 or Tyk2) and Rack-1 were transfected into 293T cells, Rack-1 was translocated to the perinuclear region (Fig. 7). This perinuclear translocation was not observed when Jak1 KE or Jak2 KE was cotransfected with Rack-1, indicating that the kinase activity of Jaks is required for the translocation of Rack-1. As Tyk2 phosphorylated Rack-1, and as the kinase activity was essential for the perinuclear translocation of Rack-1, it was possible that phosphorylated Rack-1 might translocate to the perinuclear region. To test this hypothesis, we transfected Tyk2 and the Rack-1 E mutant, which lacks tyrosine 194, the residue that is phosphorylated by Jaks. Surprisingly, the Rack-1 E mutant translocated to the perinuclear region in a similar manner as wild-type Rack-1 (Fig. 7). One possible explanation for this observation is that Tyk2 might phosphorylate another target, which forms a complex with Rack-1, and translocates Rack-1 to the perinuclear region. Another possible explanation is that Tyk2 might phosphorylate endogenous Rack-1 in 293T cells, this phosphorylated endogenous Rack-1 might form a complex with the transfected Rack-1 E mutant, and the two types of Rack-1 might then be translocated to the perinuclear region together. To determine the precise mechanism of Rack-1 translocation, similar analysis must be performed using Rack-1 null cells.

In summary, we have demonstrated that Rack-1 associates with Jaks. Specifically, Rack-1 interacts with the pseudokinase and kinase domains of Tyk2. Two regions of Rack-1, the N terminus and the middle portion of the protein (aa 138–203), contribute to binding Tyk2. In addition, we have shown that tyrosine 194 of Rack-1 is phosphorylated by Jaks. Neither the phosphorylation state of Rack-1 nor of the Jak has an influence on the association of the two proteins. Furthermore, Rack-1 is translocated to the perinuclear region by the activation of Jaks.

The function of Rack-1 in cytokine signaling is still unclear. Because Rack-1 is a WD repeat-containing protein with no enzymatic activity (20), and because Rack-1 binds to PKC, Src homology 2-containing proteins such as Src, phospholipase Cγ, and *ras*-GTPase-activating proteins (21, 22), it has been reported that Rack-1 functions as a scaffold protein that recruits specific signaling elements. Rack-1 also binds to the IFNR β-chain (13), Stat1 (15), and, as we have shown here, Jaks. It is possible that Rack-1 functions as a scaffold protein that aligns the signaling molecules in the cytokine-signaling cascade. Additionally, the fact that Rack-1 is phosphorylated by Jaks and is translocated to the perinuclear region by activation of Jaks raises the possibility that Rack-1 functions as a signaling molecule in the cytokine signaling cascade.

Acknowledgments

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Human cord blood- and bone marrow-derived CD34$^+$ cells regenerate gastrointestinal epithelial cells

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ABSTRACT

In the present study, we aimed to clarify the capacity of human cord blood- and bone marrow-derived progenitor cells to generate gastrointestinal epithelial cells in clinical and experimental transplantation settings. First, in a clinical transplantation setting, gastrointestinal tissues derived from female pediatric or juvenile recipients of allogeneic sex-mismatched bone marrow and cord blood transplantation were examined for the presence of donor-derived epithelial cells. Gastrointestinal specimens of allogeneic recipients included Y chromosome$^+$ cytokeratin$^+$ epithelial cells at a frequency of 0.4–1.9%. To further determine the capacity of purified human progenitor cells, human cord blood- or bone marrow-derived CD34$^+$ cells were transplanted into newborn NOD/SCID/β2-microglobulin^{null} mice as an experimental transplantation assay. When gastrointestinal tissues derived from recipient mice were subjected to FISH and immunofluorescence analyses, human epithelial cells were identified at a frequency of 0.24–0.58% at 3 months posttransplantation. Finally, double FISH analyses using species-specific probes revealed that human chromosome$^+$ epithelial cells did not possess any murine chromosomes, indicating that donor-derived epithelial cells were not generated only by cell fusion. On the basis of these findings, it is concluded that purified human cord blood and bone marrow CD34$^+$ progenitor cells can generate gastrointestinal epithelial cells across allogeneic and xenogeneic histocompatibility barriers.

Key words: stem cell • plasticity • fusion • epithelium

At the very end of the 20th century, Petersen et al. (1) reported that bone marrow (BM) cells possessed an extensive capacity to generate hepatocytes as well as hematopoietic cells. The notion that BM-derived progenitor cells are capable of generating