有意差が強く，STAT4（OR：1.65，$p=4.67\times10^{-5}$），interferon（IFN）regulatory factor-transportin（IRF-TNPO）（OR：1.52，$p=1.52\times10^{-7}$）も有意差を示した．

その後カナダの同じグループから，前回のGWASで$p<1\times10^{-4}$であった遺伝子について，欧州と北米の症例を加えて解析した結果として，新たにIRF-TNPO3（OR：1.57，$p=8.66\times10^{-13}$），17q12-21〔zona pellucida binding protein 2（ZPBP2）〕（OR：0.72，$p=3.50\times10^{-13}$），membrane metallo-endopeptidase-like 1（MMEL1）（OR：1.33，$p=3.15\times10^{-8}$）がPBCの疾患感受性遺伝子として，“*Nat Genet*”誌に2010年に報告された[2]．また，同号の“*Nat Genet*”誌に発表されたイタリアのグループの解析結果は，カナダから発表されたIL12とIL12RB2のPBC発症への関連をreplicationするとともに，新たにSPIB（OR：1.46，$p=7.9\times10^{-11}$），IRF-TNPO3（OR：1.63，$p=2.8\times10^{-10}$），17q12-21〔IKAROS family zinc finger 3（IKZF3）〕（OR：1.38，$p=1.7\times10^{-10}$）をPBCの疾患感受性遺伝子として同定したものであった[3]．

2011年にはイギリスのグループから，1,840例のPBC患者と5,163例のWellcome Trust Case Control Consortiumのコントロールを対象とした507,467 SNPのGWASの結果が，“*Nat Genet*”誌に発表された[4]．それまでに報告されていた欧米人で有意な遺伝子多型の多くがreplicationされるとともに，新たにPBC発症に関連する10以上の遺伝子多型が同定された．

以上のように，現在までにGWASで有意水準に達したPBC疾患感受性遺伝子として，HLA以外に21の遺伝子座が報告されている（**表1**）．

4 PBC発症に関連するshared autoimmune susceptibility loci

現在までにGWASで報告されたHLA以外のPBC疾患感受性遺伝子の21遺伝子座のうち，11遺伝子座（11/21＝52.4%）はすでにほかの自己免疫疾患で報告されていた疾患感受性遺伝子座（shared autoimmune susceptibility loci）に相当する（**表1**）．これらの自己免疫疾患には，多発性硬化症（multiple sclerosis：MS），クローン病（Crohn's disease：CD），潰瘍性大腸炎（ulcerative colitis：UC），セリアック病（celiac disease），関節リウマチ（rheumatoid arthritis：RA），全身性硬化症（systemic sclerosis：SSc），全身性エリトマトーデス（systemic lupus erythematosus：SLE），Sjögren症候群（Sjögren syndrome：SjS），I型糖尿病（type 1 diabetes：T1D），気管支喘息（asthma），乾癬（psoriasis），強直性脊椎炎（ankylosing spondylitis：AS）などの多数の疾患感受性遺伝子が含まれている．PBC発症にもほかの自己免疫疾患と共通したdisease pathwayが関与していることが強く示唆されるが，なかでもshared autoimmune susceptibility lociの11 lociの内，9 lociが消化管の粘膜免疫機構の異常が原因と考えられているCD，UC，セリアック病の疾患感受性遺伝子に一致することは，PBCの発症に粘膜免疫機構の異常が関与している可能性を示唆しており，きわめて興味深い．

5 PBC発症に関連するshared autoimmune-disease pathway

IL12/IL12Rによるシグナル伝達は，抗原提示細胞からSTAT4の活性化を介してIFNγの産生を誘導し，①T細胞のT helper（Th）1細胞への分化，②IL23によるIL17産生ヘルパーT（Th17）細胞の誘導の制御に関与している．またIL12A，IL12RB，STAT4の遺伝子多型は，炎症性腸疾患〔inflammatory bowel disease：IBD（CD，UC，セリアック病）〕だけでなくMS，RA，SLE，SSc，SjS，T1D，乾癬，ASなどの広範な自己免疫疾患発症との関連が報告されている．欧米人PBCにおいて，IL12/IL12Rの遺伝子多型がPBC発症と最も強い関連を示したことから，IL12シグナル伝

表 1. GWASにより報告されたPBCの疾患感受性遺伝子

遺伝子	染色体	機能	報告			疾患感受性遺伝子として報告されている PBC 以外の自己免疫性疾患 ④
			カナダ ①	イタリア・カナダ ②	イギリス ③	
HLA-DQB1	6p21.3	抗原提示	○	○	○	多くの自己免疫性疾患
IL12A	3q25.33-q26	IL12 シグナル伝達，Th1 細胞への分化	○	○	○	セリアック病，MS
IL12RB2/SCHIP1	1p31.2	IL12 シグナル伝達，Th1 細胞への分化	○	○	○	乾癬，CD，UC，AS，SSc，BD
STAT4	2q32	IL12 シグナル伝達，Th1 細胞への分化			○	RA，SLE，SjS，SSc，乾癬
IRF5/TNPO3	7q32.1	TLR-IFN シグナル伝達		○	○	SLE，RA，SSc，SjS，UC
IKZF3-ZPBP2-GSDMB-ORMDL3	17q12-21	B 細胞の分化・アポトーシス，上皮細胞の分化・アポトーシス，小胞体ストレスの制御　など		○	○	気管支喘息，CD，T1D，UC
MMEL1	1p36	membrane metallo-endpeptidase-like 1, ペプチド結合を切断		○	○	RA，セリアック病，MS
SPIB	19q13	B 細胞の分化		○	○	none
DENND1B	1q31	guanine exchange factor（GEF）for RAB35, 貪食に関連			○	小児喘息，CD
CD80	3q13	T 細胞の共刺激			○	セリアック病，JIA，AD
IL7R	5p13	B 細胞・T 細胞の分化			○	MS，UC
CXCR5	11q23	BLC の受容体，リンパ球の遊走，接着			○	none
TNFRSF1A	12p13	TNFα 受容体，TNFα-NFκB シグナル伝達，アポトーシス			○	MS
CLEC16A	16p13	C type lectin containing family, 機能の詳細は不明			○	MS，RA，CD，T1D，セリアック病
NFKB1	4q24	ストレス，サイトカインなどのさまざまな刺激に迅速に応答してさまざまな遺伝子の転写を制御			○	noine
RAD51L1	14q24	DNA 修復			○	none
MAP3K7IP1（*TAB1*）	22q13	IL1/TLR-NFκB シグナル伝達，TGFβ シグナル伝達			○	none
未知	7p14	不明			○	none
未知	16q24	不明			○	none
PLCL2	3p24	B 細胞受容体からのシグナル伝達の負の制御			○	none
RPS6KA4	11q13	TLR 刺激によるサイトカイン産生の抑制			○	none
TNFAIP2	14q32	TNFα induced protein 2，機能の詳細は不明			○	none

①：文献 1），②：文献 2），3），③：文献 4），④：2007 年 1 月～2012 年 2 月に報告されている疾患

○：それぞれの論文で報告された疾患感受性遺伝子

AT：autoimmune thyroiditis（自己免疫性甲状腺炎），BD：Behcet's disease（ベーチェット病），JIA：juvenile idiopathic arthritis（若年性特発性関節炎），AD：atopic dermatitis（アトピー性皮膚炎）

達に関連した免疫制御系の遺伝的多型が，Th1細胞やTh17細胞の制御異常を介して，PBCの発症と強く関連している可能性が示唆される．PBCマウスモデルであるdominant negative form of transforming growth factor β type Ⅱ（dnTGFβⅡ）マウスからIL12p40を欠損させると胆管炎が発症しなくなることも，胆管炎発症にIL12シグナル伝達が重要であることを示唆している[11]．

PBCの発症に関して，腫瘍壊死因子（tumor necrosis factor：TNF）やToll-like receptor（TLR）の関与が推測されていたが[12]，GWASにおいてもTNF/TLR- nuclear factor（NF）κBシグナル伝達に関連した多くの遺伝子座が同定された〔i.e. IRF/TNPO，TNF receptor superfamily member 1A（TNFRSF1A），NFκB1，mitogen-activated protein kinase kinase kinase 7-interacting protein 1（MAP3K7IP1），TNF α-induced protein 2（TNFAIP2）〕．これらの多くはほかの自己免疫疾患ですでに報告されていたshared autoimmune susceptibility loci（**表1**）であり，TNF/TLR-NFκB経路を介した免疫反応やアポトーシスの制御の重要性を示唆しているものと思われる．

B細胞の成熟や分化に関連した遺伝子座〔IL7R，IKZF3，SPIB，phospholipase C-like 2（PLCL2）〕も，疾患感受性遺伝子として同定されている．IL7RはpreB細胞の増殖に，IKZF3は長期B細胞記憶に重要な役割を果たす形質細胞への分化に，SPIB，PLCL2はB細胞受容体からのシグナル伝達に関連する遺伝子であり，これらの遺伝子多型が疾患感受性と有意に相関することは，PBCにおいてもB細胞が自己抗体産生や抗原提示，T細胞-B細胞相互作用を介してPBCの発症に関与していることを示唆している．

その他，上皮細胞の分化やアポトーシス，小胞体ストレスに関連した遺伝子領域（chromosome 17q12-21）も疾患感受性遺伝子として報告されており，これらは気管支喘息やIBDの疾患感受性遺伝子でもあることから，上皮障害の重要性を示唆しているもと思われる．

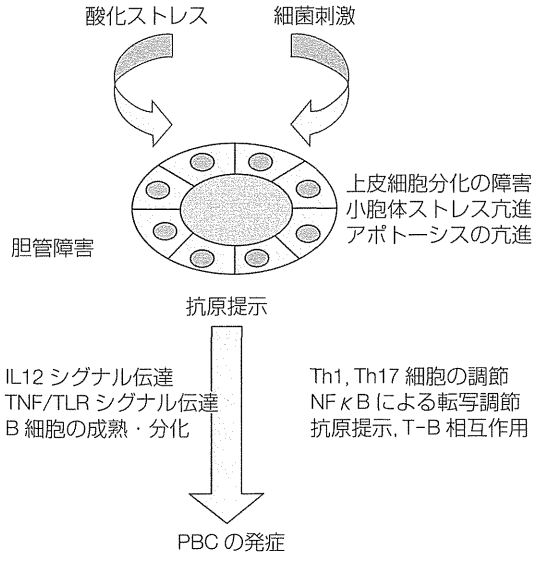

図1．GWASの結果から推定されるPBC発症機構

おわりに

欧米人PBCのGWASから，①HLAがPBCの最も強い疾患感受性遺伝子であること，HLA領域以外の遺伝子では，②IL12/IL12Rシグナル伝達，②TLR/TNFα-NFκBシグナル伝達，③B細胞の成熟・分化，④上皮細胞の分化・アポトーシスなどに関連する遺伝子多型のPBC発症における重要性が明らかとなった．また，これらの遺伝子多型の多くは，ほかの自己免疫疾患の発症との関連が報告されている遺伝子多型と重複しており（shared autoimmune susceptibility loci），PBCの発症がほかの自己免疫疾患と同様に，免疫関連分子や上皮の分化・アポトーシスに関連した遺伝的素因の影響を受けていることが示唆される（**図1**）．

今後はGWASを用いて日本人PBCの疾患関連遺伝子を同定するとともに，欧米で報告されている疾患感受性遺伝子と比較検討することにより，疾患発症に至るdisease-pathwayを明らかにする必要がある[13]．また，治療反応性，病理学的活動性，自己抗体の産生プロフィール，転帰など，

PBCの病態・病型分類にもとづく層別化解析をおこない，PBCの病態形成，進展に関する分子標的を同定し，治療抵抗性の症例に対する新しい治療法を開発する必要がある[14)~17)]．

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Significance of Immunoglobulin G4 (IgG4)-Positive Cells in Extrahepatic Cholangiocarcinoma: Molecular Mechanism of IgG4 Reaction in Cancer Tissue

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IgG4 reactions consisting of marked infiltration by immunoglobulin G4 (IgG4)-positive plasma cells in affected organs is found in cancer patients as well as patients with IgG4-related diseases. Notably, extrahepatic cholangiocarcinomas accompanying marked IgG4 reactions clinicopathologically mimic IgG4-related sclerosing cholangitis. The regulatory cytokine interleukin (IL)-10 is thought to induce the differentiation of IgG4-positive cells. In this study, to clarify the mechanism of the IgG4 reaction in extrahepatic cholangiocarcinoma, we investigated nonprofessional antigen-presenting cells (APCs) generating IL-10–producing regulatory T cells (anergy T cells) and Foxp3-positive regulatory cells producing IL-10. Immunohistochemistry targeting IgG4, HLA-DR, CD80, CD86, and Foxp3 was performed using 54 cholangiocarcinoma specimens from 24 patients with gallbladder cancer, 22 patients with common bile duct cancer, and eight patients with cancer of the Papilla of Vater. Moreover, a molecular analysis of Foxp3 and IL-10 was performed using a cultured human cholangiocarcinoma cell line. Consequently, 43% of the cholangiocarcinomas were found to be abundant in IgG4. Those expressing HLA-DR but lacking costimulatory molecules (CD80 and CD86) and those expressing Foxp3 detected by an antibody recognizing the N terminus accounted for 54% and 39% of cases, respectively. Moreover, the number of IgG4-positive cells was larger in these cases than in other groups. In cultured cells, the presence of a splicing variant of Foxp3 messenger RNA and the expression of IL-10 were demonstrated. *Conclusion*: Extrahepatic cholangiocarcinoma is often accompanied by significant infiltration of IgG4-positive cells. Cholangiocarcinoma cells could play the role of nonprofessional APCs and Foxp3-positive regulatory cells, inducing IgG4 reactions via the production of IL-10 indirectly and directly, respectively. (HEPATOLOGY 2012;56:157-164)

Biliary tract cancers can be anatomically divided into intrahepatic and extrahepatic cholangiocarcinomas, the latter including hepatic hilar cancer, common bile duct cancer, gallbladder cancer, and cancer of the Papilla of Vater. The biological behavior and carcinogenesis of each cancer differ, but the histology of most biliary tract cancers is the same as that of ordinary adenocarcinomas. In addition to neoplastic lesions, several types of cholangitis causing biliary stenosis are important in the differential diagnosis of biliary diseases. Particularly, primary sclerosing cholangitis and a complication of immunoglobulin G4 (IgG4)-related systemic diseases, IgG4-related sclerosing cholangitis, clinicopathologically mimic extrahepatic cholangiocarcinomas.

IgG4 is a minor immunoglobulin subtype composing 3%-6% of all the IgG circulating in adults,[1] but is important for a systemic disorder, IgG4-related disease, that features elevated serum IgG4 levels and abundant

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; HPF, high-power field; IgG4, immunoglobulin G4; IL, interleukin; MHC-II, major histocompatibility complex class II; mRNA, messenger RNA; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; Treg, regulatory T cell.

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infiltration with IgG4-positive plasma cells in affected organs.[1-3] Moreover, IgG4-related cholangitis and pancreatitis (autoimmune pancreatitis, type 1) are characterized by sclerosing lesions (storiform fibrosis) and cholangiocarcinomas and pancreatic cancer usually accompany some degree of desmoplastic change and also, in some cases of pancreatic cancer, IgG4 reactions.[4] Therefore, a pathological examination is necessary to differentiate IgG4-related diseases from tumors in pancreaticobiliary lesions. We have already observed that extrahepatic cholangiocarcinomas also accompany various degrees of IgG4 reactions assumed to be associated with the evasion of immunosurveillance (Kimura et al., unpublished data). However, the mechanisms inducing IgG4 reactions in cholangiocarcinoma tissue are still unknown.

Interleukin (IL)-10, a regulatory cytokine mainly produced by Foxp3+ regulatory T cells (Treg cells), T helper 2 cells, and IL-10–producing Treg cells, is thought to induce the differentiation of IgG4-positive plasma cells or favor B cell switching to IgG4 in the presence of IL-4.[5,6] The expression of Foxp3 and IL-10 has been demonstrated in several carcinoma tissues and cultured cancer cell lines, suggesting that cancer cells themselves induce the Treg cell–like immunoregulatory milieu to evade immunosurveillance.[7-10].

Major histocompatibility complex class II (MHC-II)–positive cells lacking the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) induce anergy to native T cells. Among T cell subsets, Treg type 1 cells characterized by the production of IL-10 are induced by immature dendritic cells (DCs).[11] Moreover, costimulation-dependent T cell clones stimulated without provision of the costimulatory signal were demonstrated not to be proliferative, but to differentiate into IL-10–producing anergic T cells in primary biliary cirrhosis.[12] In addition to immunocompetent cells such as DCs, nonimmunocompetent cells, including carcinoma and normal epithelial cells, have been demonstrated to express MHC-II, indicating an ability for antigen presentation, but these MHC-II–positive epithelial cells are usually called nonprofessional antigen-presenting cells (APCs), differing from professional APCs such as DCs. Several studies have suggested that antigen presentation by MHC-II–positive epithelial cells that lack costimulation signals, such as keratinocytes and pancreatic islet cells, would favor the generation of anergic T cells.[13-15]

It is clinicopathologically important, but practically difficult, to differentiate between IgG4-related sclerosing cholangitis and extrahepatic cholangiocarcinoma. In this study, we retrospectively evaluated IgG4-positive plasma cells in extrahepatic cholangiocarcinomas and mechanisms in terms of cholangiocarcinoma cells as nonprofessional APCs and regulatory cells. This study should help to clarify the pathological significance of IgG4 reactions in cholangiocarcinomas and also IgG4-related diseases.

Patients and Methods

Patients and Tissue Preparations. Formalin-fixed and paraffin-embedded sections of 54 surgically resected specimens from 24 gallbladder cancers, 22 common bile duct cancers, and eight cancers of the Papilla of Vater (29 men, 25 women; average age, 74 years) were obtained from the registry of liver diseases in the Department of Pathology, Kanazawa University School of Medicine. Each cholangiocarcinoma was classified histologically as a well-differentiated (including papillary), moderately differentiated, or poorly differentiated adenocarcinoma based on the predominant histological grade. Special histological types such as adenosquamous carcinoma and mucinous carcinoma were not included in the present study. Serial sections (4 μm) were prepared from each formalin-fixed, paraffin-embedded block.

Immunohistochemistry. The deparaffinized and rehydrated sections were microwaved in citrate buffer (pH 6.0) for CD80 and CD86 or ethylene diamine tetraacetic acid buffer (pH 9.0) for Foxp3 for 20 minutes in a microwave oven. Following the blocking of endogenous peroxidase activity, these sections were incubated at 4°C overnight with antibodies against IgG4 (mouse monoclonal; diluted 1:200; Southern Biotech, Birmingham, AL), Foxp3 that reacts with the C terminus (mouse monoclonal; 5 μg/mL; Abcam, Tokyo, Japan), Foxp3 that reacts with the N terminus (rat monoclonal, 2.5 μg/mL, eBioscience, San Diego, CA), HLA-DR (mouse monoclonal, 0.5 μg/mL, Dako Japan, Tokyo), CD80 (rabbit monoclonal, 1:200, Epitomics, Burlingame, CA), and CD86 (rabbit monoclonal, 1:250, Abcam, Tokyo, Japan) and then at room temperature for 1 hour with anti-mouse, anti-rabbit, or anti-goat immunoglobulin conjugated to a peroxidase-labeled dextran polymer (Simple Staining Kit; Nichirei, Tokyo, Japan). After a benzidine reaction, sections were counterstained lightly with hematoxylin. No positive staining was obtained when the primary antibodies were replaced with an isotype-matched, nonimmunized immunoglobulin as a negative control of the staining procedures.

Histological Examination. In addition to the histological observations by hematoxylin and eosin staining, the distribution of the immunopositive cells was

examined. In a primary survey, we examined all tumorous areas in each specimen and, for counting IgG4-positive mononuclear cells, selected three representative areas containing IgG4-positive plasma cells, and expressed the results as the mean number of immunopositive cells in high-power fields (HPFs). Because ≥10 IgG4-positive cells/HPF is proposed according to HISORt (Histology, Imaging, Serology, Other organ involvement, Response to therapy) criteria published for autoimmune pancreatitis,[16,17] the cases with ≥10 and <10 IgG4-positive cells/HPF on average were evaluated as IgG4-rich and IgG4-poor cases, respectively. For the expression of Foxp3, HLA-DR, CD80, and CD86, positive carcinoma cells were evaluated as positive (distinct expression) or negative (no or faint expression) according to the staining intensity.

Cultured Cells. Two commercially available cell lines, HuCCT1 and MCF7 (positive control of IL-10),[10] were obtained from Health Science Research Resources Bank (Osaka, Japan). The cell lines were derived from cholangiocarcinoma and breast cancer cells, respectively.

Reverse-Transcription Polymerase Chain Reaction. The cell lines were cultured in flasks with a standard medium for 48 hours. Cultured cells were collected from the flasks or plates with a cell scraper for determination of the baseline messenger RNA (mRNA) expression of Foxp3 and IL-10 by via reverse-transcription polymerase chain reaction (RT-PCR). Lymph node tissue was also used as a positive control for Foxp3 mRNA. Briefly, total RNA was isolated from each sample with the RNeasy Total RNA System (QIAGEN, Hilden, Germany) and treated with RNase-Free DNaseI. For RT-PCR, 1 μg of total RNA, M-MLV RTase (ReverTra Ace, Toyobo, Tokyo, Japan) and oligo-dT primers were used. Polymerase chain reaction (PCR) amplification was performed using DNA polymerase (Takara EX Taq, Takara, Tokyo, Japan) and specific primers for human mRNA sequences (Table 1). The glyceraldehyde 3-phosphate dehydrogenase mRNA was used as a housekeeping gene. Following After PCR, an annealing of primers for 1 minute, and an extension at 72°C for 2 minutes (the annealing temperature and cycle number are shown in Table 1), PCR products were subjected to agarose gel electrophoresis.

Enzyme-Linked Immunosorbent Assay. Approximately 1×10^4 HuCCT1 cells per well in 96-well plates were cultured for 24 hours. Supernatants were then tested for human IL-10 via enzyme-linked immunosorbent assay (ELISA) (R&D Systems).

Statistical Analysis. Data were analyzed using the Welch t test; $P < 0.05$ was considered statistically significant.

Table 1. Primers Used for RT-PCR

Transcript	Primers	Product Size
Foxp3		
Exon 1	Forward: 5′-ACCGTACAGCGTGGTTTTTC-3′	111 bp
	Reverse: 5′-AGGCTTGGTGAAGTGGACTG-3′	
Exon 3	Forward: 5′-TGCCTCCTCTTCTTCCTTGA-3′	125 bp
	Reverse: 5′-GGAGGAGTGCCTGTAAGTGG-3′	
Exons 10-12	Forward: 5′- CACAACATGCGACCCCCTTTCACC -3′	167 bp
	Reverse: 5′- AGGTTGTGGCGGATGGCGTTCTTC-3′	
Exon 12	Forward: 5′- CAGCTGCTCGCACAGATTAC -3′	91 bp
	Reverse: 5′- TTGGGGTTTGTGTTGAGTGA-3′	
IL-10	Forward: 5′- TGCAAAACCAAACCACAAGA -3′	325 bp
	Reverse: 5′- GCATCACCTCCTCCAGGTAA-3′	
GAPDH	Forward: 5′-GCACCGTCAAGGCTGAGAAC-3′	142 bp
	Reverse: 5′-ATGGTGGTGAAGACGCCAGT-3′	

Abbreviations: bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-10, interleukin 10; RT-PCR, reverse-transcription polymerase chain reaction.

Results

Infiltration of IgG4-Positive Cells in Extrahepatic Cholangiocarcinoma. Immunohistochemistry revealed that IgG4-positive plasma cells were scattered within and around cancerous nests to various degrees in most cases (Fig. 1). In the cases with marked infiltration, the IgG4-positive cells were prominent with intermingling of other inflammatory cells. Figure 1C shows the number of IgG4-positive cells/HPF in extrahepatic cholangiocarcinomas from common bile ducts, gallbladder, and the Papilla of Vater, but there was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinomas. Therefore, they were integrated as shown in Fig. 1D. Consequently, the combined quantitative evaluation revealed that 23 (43%) of 54 cholangiocarcinoma patients had ≥10 IgG4-positive cells/HPF. There was no correlation between the density of IgG4-positive cells and any clinicopathological factor including age, sex, anatomical location (common bile ducts, gallbladder, and the Papilla of Vater), or the histological differentiation (well, moderate, and poor) of extrahepatic cholangiocarcinoma.

Cholangiocarcinoma Cells as Nonprofessional APCs and Their Association with IgG4 Reactions. Representative images of immunostaining are shown in Fig. 2. Expression of HLA-DR was found in some infiltrating immunocompetent cells. Moreover, HLA-DR–positive cholangiocarcinoma cells were also found in 33 of 54 cases. HLA-DR expression in tumor cells showed uniformity and metastatic foci in lymph nodes as well as main tumors expressing HLA-DR. In contrast, the expression of costimulatory molecules (CD80 and CD86) was mostly faint or absent. Only four cases were clearly positive for CD86 in cholangiocarcinoma

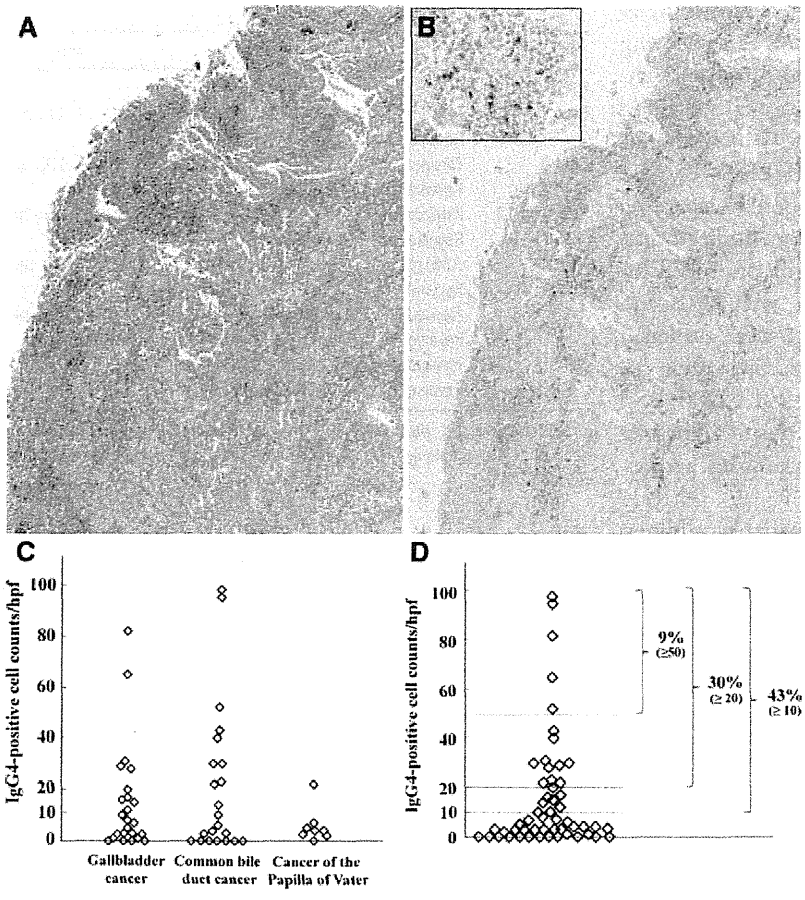

Fig. 1. IgG4-positive cells in extrahepatic cholangiocarcinomas. (A) Gallbladder cancer. A papillary adenocarcinoma with prominent inflammatory cells was found (original magnification: ×40). (B) Immunohistochemistry for IgG4. Numerous IgG4-posituve cells are present in the inflamed stroma (original magnification: ×40). The inset shows a higher magnification (original magnification: ×400). (C) Number of IgG4-positive cells/HPF in common bile duct cancer, gallbladder cancer, and cancer of the Papilla of Vater. There was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinoma. (D) Number of IgG4-positive cells in cholangiocarcinoma. A quantitative evaluation revealed that 23 (43%), 16 (30%), and five (9%) of 54 cholangiocarcinoma patients had ≥10, ≥20, and ≥50 IgG4+ cells/HPF, respectively.

cells, and all of them were positive for HLA-DR. No cases evidently expressed CD80. Cholangiocarcinoma cells expressing HLA-DR but lacking costimulatory molecules (CD80 and CD86) were found in 29 of 54 cases (54%) and suggested to act as nonprofessional APCs inducing IL-10–producing anergy T cells. The relation between IgG4 reactions and HLA-DR and costimulatory molecules in cancer cells is shown in Fig. 3. In cases of positivity for HLA-DR and negativity for costimulatory molecules, the number of IgG4-positive cells was significantly higher than in cases of negativity for HLA-DR and of positivity for both HLA-DR and costimulatory molecules.

Cholangiocarcinoma Cells as Regulatory Cells. Immunohistochemistry using the antibody reacting with the C terminus of Foxp3 detected only mononuclear cells (Treg cells), but the antibody reacting with the N terminus highlighted cholangiocarcinoma cells as well as Treg cells (Fig. 4A). The cytoplasm as well as nucleus of tumor cells was positive in several cases. However, because Foxp3 is a transcription factor, the nuclear pattern was evaluated as functional expression. Consequently, 21 of 54 (39%) cholangiocarcinomas tested positive for Foxp3 by the antibody reacting with the N terminus. The relation between the IgG4 reaction and Foxp3 expression in cholangiocarcinoma cells is shown in Fig. 5. In cases of positivity for Fopx3, the number of IgG4-positive cells was significantly higher than in cases of negativity for Foxp3.

RT-PCR analysis demonstrated that a cholangiocarcinoma cell line, HuCCT1, expressed the mRNA of Foxp3, but close examination using four sets of primers corresponding to exons 1, 3, 10-12, and 12 revealed a lack of exon 3 (Fig. 6), suggesting the presence of a splicing variant of Foxp3 in cholangiocarcinoma cells. Moreover, RT-PCR and ELISA revealed that HuCCT1 cells expressed IL-10 mRNA (Fig. 6) and protein in the culture medium at 7.8-15.6 pg/mL.

Discussion

IgG4 is important to the pathogenesis of IgG4-related diseases. However, patients with pancreatic adenocarcinomas accompanying IgG4 reactions and/or

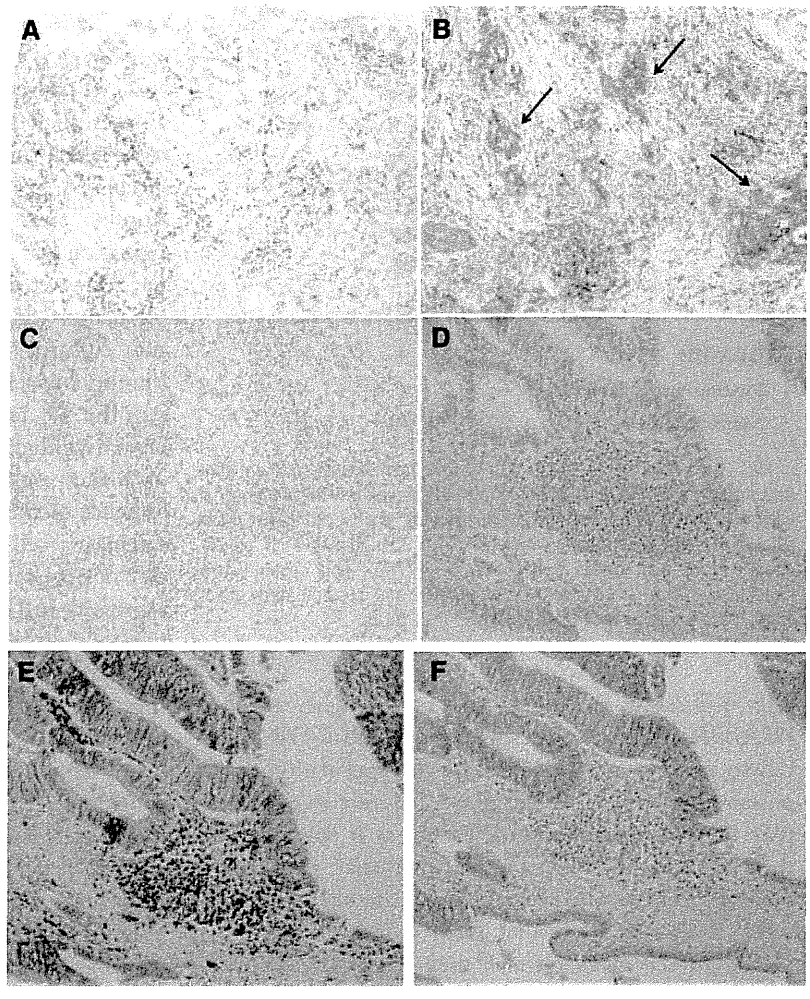

Fig. 2. Immunohistochemistry for IgG4 (A,D), HLA-DR (B,E), CD80 (C), and CD86 (F). (A-C) IgG4-rich case of gallbladder cancer. Numerous IgG4-positive cells were found within cancer tissue (A). In addition to infiltrating mononuclear cells, carcinoma cells also tested positive for HLA-DR (B, arrows). No tumor cells were positive for CD80 (C). (D-F) IgG4-poor case of common bile duct cancer. No IgG4-positive cells were found (D), but obvious expression of HLA-DR and CD86 in carcinoma cells was found (E,F). Original magnification: ×200.

elevated serum IgG4 levels[4,18-20] and with pancreatic and biliary cancers arising from IgG4-related diseases[20-22] have been reported, though a cause-and-effect relationship between IgG4 reactions and cancers has yet to be demonstrated. Moreover, in IgG4-nonrelated diseases, including primary sclerosing cholangitis, IgG4 reactions were found to various degrees.[23,24] Therefore, the presence of IgG4-positive cells is not a histological hallmark of IgG4-related diseases, and IgG4 reactions are speculated to be nonspecific in several pathological conditions, including cholangiocarcinomas. The present study also demonstrated the presence of extrahepatic cholangiocarcinoma cases with abundant IgG4 reaction, though there was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinomas (common bile ducts, gallbladder, and the Papilla of Vater). The significance and mechanisms of IgG4 reactions in cancers as well as IgG4-related diseases are still unknown, but we speculated that cancer cells directly participate in the histogenesis of IgG4 reactions. Because the regulatory cytokine IL-10 is known to induce the differentiation of IgG4-positive plasma cells or favor B cell switching to IgG4 in the presence of IL-4,[5,6] we noted the IL-10–related regulatory cytokine network around cholangiocarcinoma tissue in this study.

Immunohistochemistry for MHC-II (HLA-DR) and costimulatory molecules (CD80 and CD86) revealed that cholangiocarcinoma cells as well as professional APCs such as B cells and DCs expressed HLA-DR and CD86. The expression of CD80 was limited in some APCs and not found in cholangiocarcinoma cells. Consequently, cholangiocarcinoma cells expressing HLA-DR, but lacking costimulatory molecules (CD80 and CD86) were found in 54% of cases. These cancer cells could act as nonprofessional APCs, possibly generating IL-10–producing Treg cells (anergy T cells), and then an IL-10–predominant cytokine milieu could cause the induction of IgG4-positive cells.[5,6] In these phenotypic cases, the number of IgG4-positive

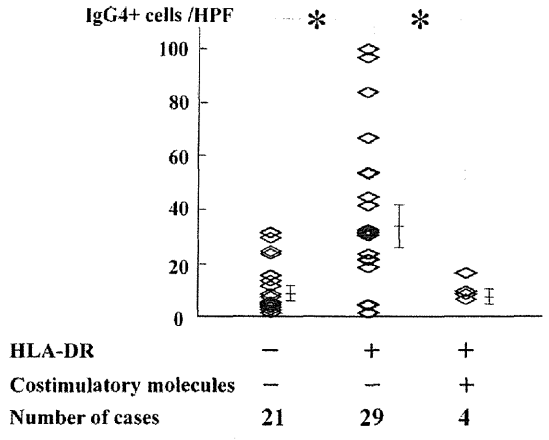

Fig. 3. Correlation between IgG4-positive cell counts and antigen-presenting-related molecules in cholangiocarcinoma. The number of IgG4-positive cells in the cholangiocarcinoma cases expressing HLA-DR but lacking costimulatory molecules (CD80 and CD86) is significantly higher than those of negativity for HLA-DR and costimulatory molecules and of positivity for both HLA-DR and costimulatory molecules. Bars indicate the mean ± SEM. $*P < 0.05$.

cells infiltrating carcinoma tissue was higher than in HLA-DR–negative cases and both HLA-DR– and CD86-positive cases, confirming this speculation. Cells positive for both HLA-DR and CD86 are suggested to play the role of professional APCs, as it was reported that MHC-II–positive thyroid epithelial cells could present antigens to T cells and activate autoreactive T cells.[25,26] Although further study is needed to clarify the functional mechanism of these cholangiocarcinoma cells as APCs, this study demonstrated that HLA-DR– and CD86-positive cancer cells were not associated with IgG4 reactions in cholangiocarcinoma tissue.

As to pathogenesis of IgG4 reactions in IgG4-related diseases, the participation of $CD4^+CD25^+Foxp3^+$ Treg cells, which are capable of producing IL-10, has been speculated.[27] Foxp3 is thought to be the master transcription factor of Treg cells and, until recently, Foxp3 expression was thought to be restricted to the T cell lineage. However, immunohistochemistry and flow cytometric analysis demonstrated that some carcinoma tissues and cultured cancer cell lines expressed Foxp3.[7-10] Immunohistochemistry using the antibody recognizing the N terminus, but not the C terminus, of Foxp3-highlighted cholangiocarcinoma tissue in 39% of cases as well as Treg cell morphology, suggesting the presence of the splicing variant of Foxp3 in cholangiocarcinoma cells. Molecular analysis using a cholangiocarcinoma cell line demonstrated that the cells expressed mRNA of Foxp3, but lack Exon 3. This type of splicing variant has already been reported in a melanoma cell line and created a novel amino acid caused by a frame shift at the C terminus.[9] This is why the antibody recognizing the C terminus of Foxp3 could not detect the variant of Foxp3 found in cholangiocarcinoma tissue. Although a functional analysis of this variant as a transcription factor is necessary, it has already been reported that Foxp3 expression is closely correlated with the expression of IL-10 in all Foxp3-positive cell lines.[10] The present study, using a cholangiocarcinoma cell line, also demonstrated that cells express mRNA of IL-10 as well as Foxp3.

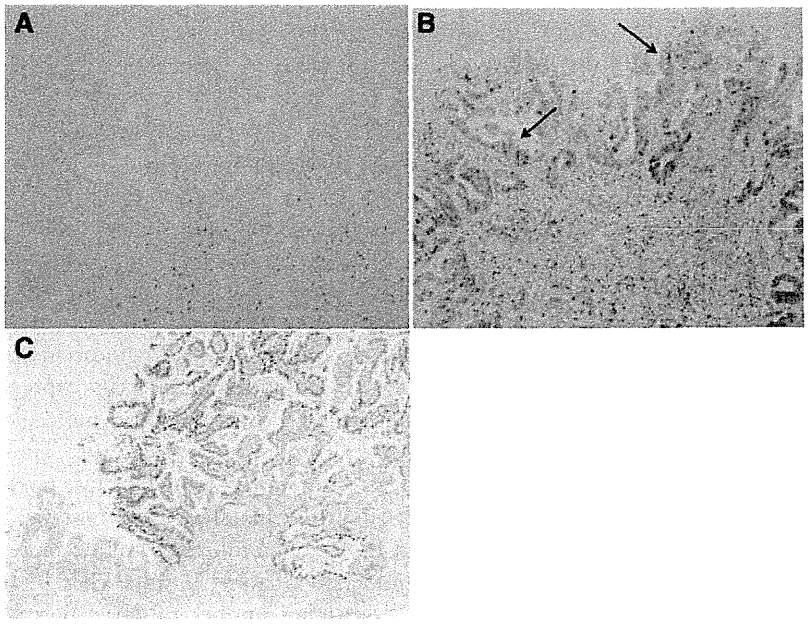

Fig. 4. Foxp3 expression in cholangiocarcinoma. Immunohistochemistry using the antibody recognizing the C terminus (A) and N terminus (B,C) of Foxp3. The antibody reacting with the C terminus detects only mononuclear cells (Treg cells) in the nuclear pattern (A). In contrast, the antibody reacting with the N terminus highlights the nucleus and cytoplasm of cholangiocarcinoma cells as well as Treg cells (B, arrows), but the localized expression in the nucleus is also found in cholangiocarcinoma cells (C). Original magnification for panels A and B is ×100; magnification for panel C is ×40.

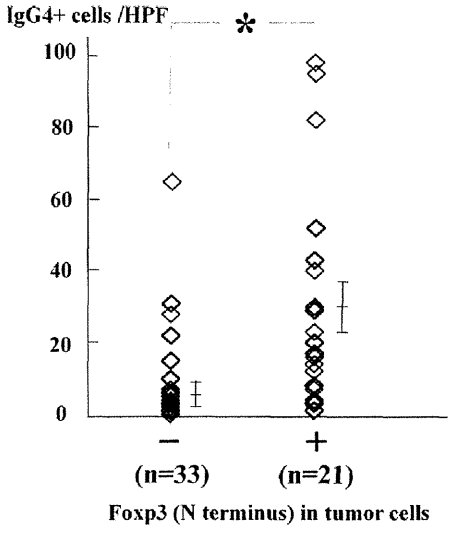

Fig. 5. Correlation between IgG4-positive cell counts and Foxp3 expression in cholangiocarcinoma. Nuclear expression of Foxp3 is found in 21 cases of cholangiocarcinoma and in these cases, the number of IgG4-positive cells was significantly higher than those of negativity for Foxp3. Bars indicate the mean ± S.E.M. *$P < 0.05$.

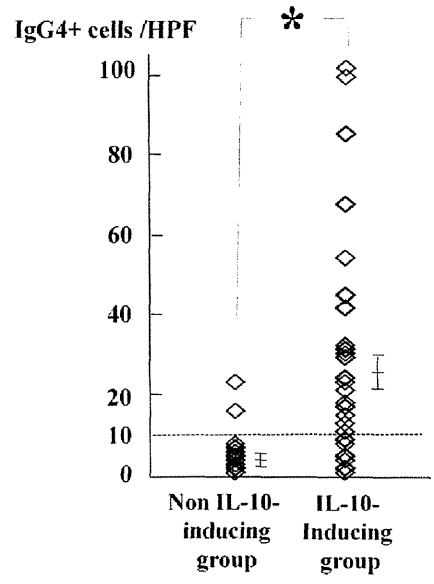

Fig. 7. Correlation between IgG4-positive cell counts and IL-10-predominant milieu. All cases were divided into two categories. The non-IL-10-inducing group includes MHC-II (HLA-DR)-negative and Foxp3-negative cases and MHC-II-positive, costimulatory molecule (CD86)-positive, and Foxp3-negative cases. The IL-10-inducing group includes MHC-II-positive and costimulatory molecule-negative cases and Foxp3-positive cases. All but two cases in the non-IL-10-inducing group were <10 IgG4+ cells/HPF, and the number of IgG4-positive cells in the IL-10-inducing group was significantly higher than that of the non-IL-10-inducing group. Bars indicate the mean ± SEM. *$P < 0.05$.

Moreover, the IL-10 protein was detected in the culture medium by ELISA at a concentration of 7.8-15.6 pg/mL, suggesting that the production of IL-10 was preserved with this splicing variant. This finding suggests that cholangiocarcinoma cells themselves function in immunosuppression similar to Treg cells via IL-10 production. This was supported by the present data that in Foxp3-positive cases, the number of IgG4-positive cells infiltrating cholangiocarcinoma tissues was higher than that in Foxp3-negative cases, though several negative cases still accompanied a significant IgG4 reaction (≥10 IgG4+ cells/HPF).

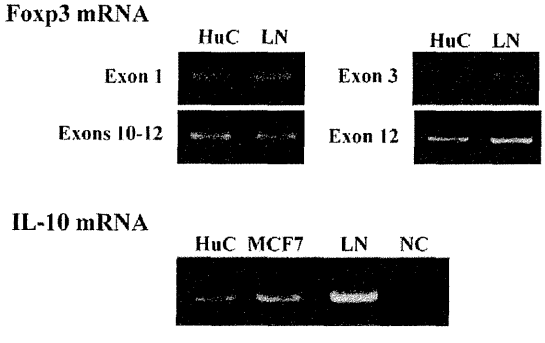

Fig. 6. Detection of Foxp3 and IL-10 mRNAs in the cultured cholangiocarcinoma cell line HuCCT1 (HuC). RT-PCR analysis using four sets of primers corresponding to exons 1, 3, 10-12, and 12 demonstrated that HuC expressed the mRNA of Foxp3, but lacked exon 3. Moreover, HuC expressed IL-10 mRNA. Each RT-PCR product yielded bands of the appropriate molecular weight. MCF7 (breast cancer cell line) and lymph node (LN) were used as positive controls, and negative control (NC) was obtained by omitting reverse transcriptase for reverse transcription of HuC.

In this study, we demonstrated two different types of IgG4 reactions in cholangiocarcinoma tissues. Although statistical significance could be obtained in terms of cholangiocarcinoma as both nonprofessional APCs and IL-10–producing regulatory cells, some cases deviated from each mechanism. Therefore, as shown in Fig. 7, we divided all cases into a non–IL-10–inducing group and an IL-10–inducing group and re-evaluated the present results accordingly. The former (n = 24) consisted of MHC-II–negative and Foxp3-negative cases and MHC-II–positive, costimulatory molecule (CD86)-positive, and Foxp3-negative cases; the latter (n = 30) included MHC-II–positive, costimulatory molecule–negative, and Foxp3-positive cases. This combined analysis demonstrated that all but two cases in the non–IL-10–inducing group were poor in IgG4 (<10 IgG4+ cells/HPF) and that the difference in IgG4 reactions between the IL-10–inducing group and the non–IL-10–inducing group was significant compared with that of the individual analysis in terms of nonprofessional APCs and IL-10–producing

regulatory cells. This finding indicates that cholangiocarcinoma cells directly participate in the induction of IgG4 reactions via an IL-10–predominent cytokine milieu as nonprofessional APCs and/or regulatory cells. However, the presence of IgG4-rich cases belonging to the non–IL-10–inducing group suggests another possible mechanism inducing IgG4 reactions in cholangiocarcinomas. Further studies are needed to clarify the mechanism of IgG4 reactions.

In conclusion, the marked infiltration of IgG-positive cells is found in several cases of cholangiocarcinoma, indicating that we should consider the differentiation of IgG4-related diseases and cholangiocarcinoma. The IgG4 reactions in cholangiocarcinomas, moreover, are closely associated with the IL-10–predominant regulatory cytokine milieu caused by cancer cells themselves directly and indirectly. Because IL-10 plays a primary role in suppressing immune responses, IgG4 reactions in cholangiocarcinoma might reflect evasion from immunosurveillance.

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Research Article

Fractalkine and Other Chemokines in Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is characterized by the autoimmune injury of small intrahepatic bile duct. On this basis, it has been suggested that the targeted biliary epithelial cells (BEC) play an active role in the perpetuation of autoimmunity by attracting immune cells via chemokine secretion. To address this issue, we challenged BEC using multiple toll-like receptor (TLR) ligands as well as autologous liver infiltrating mononuclear cells (LMNC) with subsequent measurement of BEC phenotype and chemokine production and LMNC chemotaxis by quantifying specific chemokines, specially CX3CL1 (fractalkine). We submit the hypothesis that BEC are in fact the innocent victims of the autoimmune injury and that the adaptive immune response is critical in PBC.

1. Introduction

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease recognized at histology as chronic nonsuppurative destructive cholangitis with an autoimmune pathogenesis supported by Th1 or Th17 cells producing IFN-γ or IL-17 [1, 2]. Several inflammatory cell populations, including T and B cells, are found around the affected intrahepatic bile ducts, and chemokines are believed to play a pivotal role for the infiltration of inflammatory cells [3].

A better understanding of the role of specific chemokines in liver injury is ancillary to understanding the molecular mechanisms regulating the autoimmunity process and is expected to unravel new strategies to treat PBC.

The observed patterns of chemokine expression in normal and PBC liver are illustrated in Table 1 [4]. In our recent experiments, we cultured EpCAM-positive cells (i.e., biliary epithelial cells and BEC) isolated by immunobeads from explanted liver tissue and examined the production of chemokines by protein array following the stimulation by inflammatory cytokines or Toll-like receptor (TLR) ligands [5]. Our data illustrated that BEC produce proinflammatory chemokines such as CXCL1, CXCL5, CXCL6, and CXCL8 without any specific stimulation as shown in Figure 1. On the other hand, BEC challenged with a TLR3 ligand (poly I:C) manifest a Th1 shift and the production of CCL3, CCL4, CCL5, and CXCL10. Such production of Th1 chemokines was further prompted by the interaction between CD40 on BEC and CD154 on liver infiltrating lymphocytes. Taken altogether, the evidence support the observation that BEC induces a proinflammatory environment in the absence of innate immunity stimulation and induces Th1-sifted environment when such stimulation is present.

2. Fractalkine

Fractalkine is characterized as a type-1 transmembrane molecule with the chemokine domain tethered by a 241-amino acid glycosylated stalk, a 19-amino acid transmembrane region, and 37-amino acid intracellular tail [6]. The surface-expressed transmembrane fractalkine induces the firm adhesion of leukocytes expressing its receptor CX3CR1. After shedding by the disintegrins and metalloproteinases (ADAM) 10 and 17, fractalkine also acts as a soluble leukocyte chemoattractant. Transmembrane fractalkine expressed on both endothelial and epithelial cells induces leukocyte

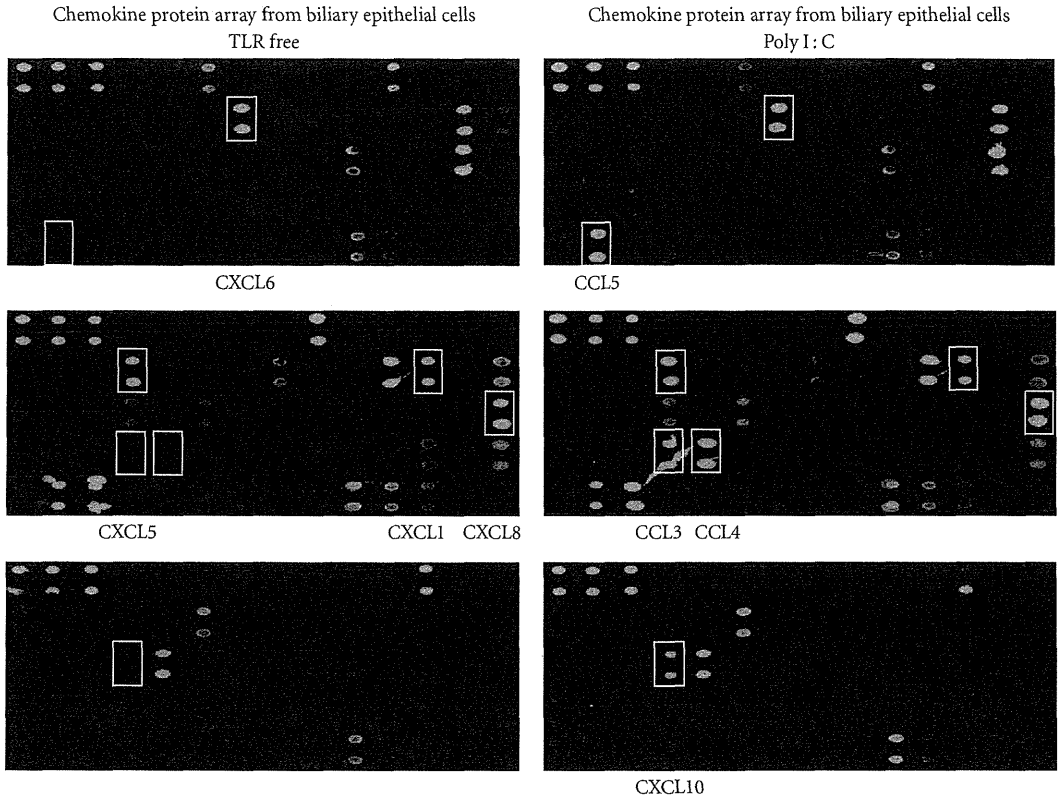

FIGURE 1: Chemokines produced by biliary epithelial cells under basal conditions or after stimulation with TLR3 ligand (poly I : C) for 48 hours. Cell-free culture supernatants were analyzed by a protein array kit to evaluate 174 different proteins simultaneously. Unstimulated cells produced detectable amounts of GRO-α/CXCL1, ENA-78/CXCL5, GCP-2/CXCL6, and IL-8/CXCL8, while poly I : C stimulation led to enhanced MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, and IP-10/CXCL10.

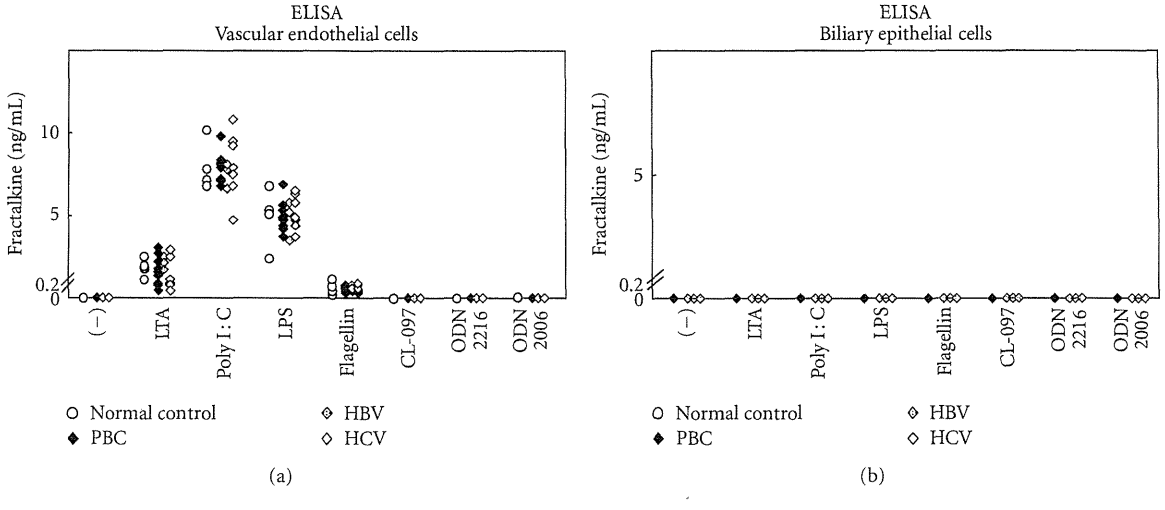

FIGURE 2: (a) Fractalkine production from endothelial cells from PBC and control (chronic hepatitis B and C) livers exposed to TLR ligands. Endothelial cells produced fractalkine with LTA, poly I : C, LPS, and flagellin with no significant differences observed between patients and control livers. (b) BEC did not produce fractalkine with any additional TLR ligand.

TABLE 1: Chemokine expression patterns in the portal tract, sinusoidal endothelium, and bile duct of normal and PBC liver.

Chemokine	Portal vein		Sinusoidal EC		Bile duct	
	Normal	PBC	Normal	PBC	Normal	PBC
CXCL9	±	+(?)	±	ND	−	+
CXCL10	±	+(?)	±	ND	−	+
CXCL11	±	ND	+	ND	ND	ND
CXCL12	−	−	−	−	+	++
CXCL16	+	+	+	+	+	++
CCL25	−	−	−	−	−	−
CCL28	−	+	−	−	−	++

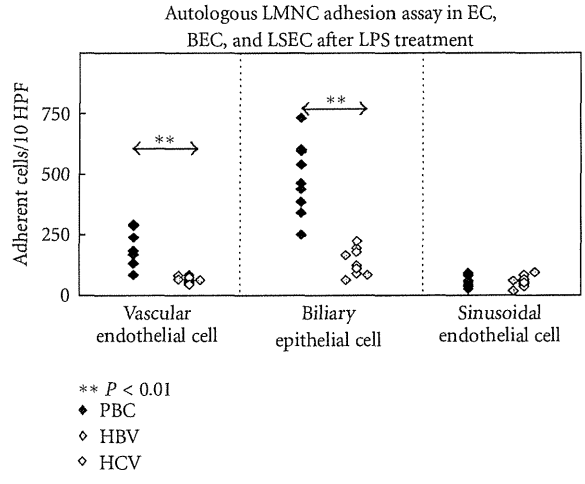

FIGURE 3: Autologous liver mononuclear cells adhesion assay using endothelial cells and BEC after stimulation with TLR4 ligand (LPS). Adherent liver mononuclear cells were stained and counted in ten random high-power microscopy fields. Liver mononuclear cells from PBC livers adhered in greater numbers than did liver mononuclear cells from controls using either endothelial cells or biliary epithelial cells, whereas liver mononuclear cells adhered only minimally to liver sinusoidal endothelial cells in all instances. Other TLR ligands did not accelerate liver mononuclear cells adhesion with neither endothelial cells nor biliary epithelial cells (data not shown).

transmigration [7]. Fractalkine is upregulated by inflammation cytokines such as TNF-α or IFN-γ, it has been proposed to contribute to inflammatory diseases by promoting the transmigration of CX3CR1-expressing cells to inflamed tissues in Crohn disease [8], rheumatoid arthritis, atherosclerosis [9], systemic lupus erythematosus [10], and most recently PBC [5]. CX3CR1 is expressed on natural killer cells, monocytes, macrophages, mucosal dendritic cells, $CD8^+$ T cells, and a subset of effector-memory $CD4^+$ T cells [11, 12]. Human Th1 cells express high levels of CX3CR1 mRNA, different from polarized Th2 cells [13, 14]. Fractalkine is expressed in limited amounts in the normal human liver, particularly near branches of the hepatic artery and in small bile ducts located at the interface between the portal tract and the hepatic lobule. In the case of acute or chronic viral hepatitis, fractalkine is detected in the areas of necrosis and inflammatory infiltration and also at the interface between the expanded portal tract and the regenerating nodule. Regenerating epithelial cells of the ductular reaction are also positive for fractalkine [15]. In kidney allograft transplantation, fractalkine is expressed in renal tubular epithelial cells, and the expression is upregulated by TNF-α, the recognized key proinflammatory cytokine in acute rejection [16]. The $CD4^+$ and $CD8^+$ T cells expressing CX3CR1 predominantly produce IFN-γ and TNF-α, and these T cells infiltrate the synovium in patients with rheumatoid arthritis [17]. In inflammatory bowel disease (IBD), intestinal microvascular endothelial cells produce high amounts of fractalkine, and IBD mucosa as well as periphery contained significantly more CX3CR1+ cells than control. Fractalkine is a major contributor to T- and monocytic-cell adhesion to endothelial cells [18]. In HCV infection, CX3CR1 is susceptible gene for hepatic fibrosis [19]. In mice models, it is unclear whether CX3CR1 positive cells are protective or trigger disease [20–25].

3. Fractalkine and PBC

Fractalkine is peripherally expressed dominantly in patients with PBC, and is upregulated in BEC of the PBC liver. CX3CR1 is expressed on infiltrating lymphocytes in the portal tracts and on intraepithelial T cells of injured bile ducts [26]. BEC manifesting senescent features in damaged

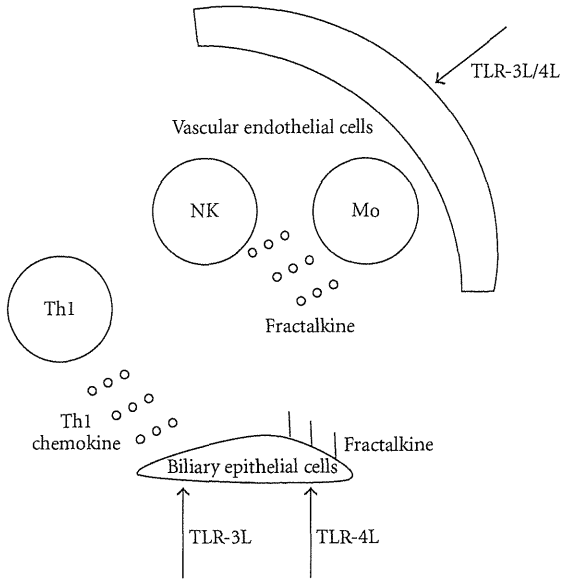

FIGURE 4: The proposed role of fractalkine is illustrated. TLR3 or TLR4 ligands stimulate vascular endothelial cells to produce fractalkine as chemokine, then fractalkine attracts CX3CR1 positive monocytes or NK cells. Subsequently, TLR4 ligand stimulated BEC produce fractalkine as cell adhesion molecule, then fractalkine recruit CX3CR1 positive cells around PBC target cells. This starts the chronic nonsuppurative destructive cholangitis and perpetuates the autoimmune pathogenesis of disease. Finally, TLR3 ligand stimulated biliary epithelial cells produce Th1 chemokines, and these chemokines are considered to contribute this autoimmune mechanism.

small bile ducts also overexpress fractalkine [27]. As previously introduced, in our recent work, we separated BEC as EpCAM positive and endothelial cells as CD31 positive by immunobeads and evaluated the production of fractalkine as chemokine by ELISA. Figure 2(a) illustrates the elevated production of fractalkine by endothelial cells challenged with TLR3 ligand (poly I:C) or TLR4 ligand (LPS). Conversely, BEC did not produce fractalkine with any other TLR ligand stimulation (Figure 2(b)), and this was not reversed with the addition of established inflammatory cytokines such as TNF-α or IFN-γ. Further, we investigated the production of fractalkine following the interaction between BEC or endothelial cells and liver infiltrating lymphocytes. As shown in Figure 3, mononuclear cells adhered with higher affinity to BEC compared to endothelial cells in the TLR4 ligand (LPS) stimulation, and this adherence was increased more in PBC than in other control diseases [5]. Fractalkine works to modulate inflammation in the BEC of PBC, thus suggesting that novel therapies to block fractalkine induced environment may prove beneficial. Based on our data, we propose a working model on the role of fractalkine as chemokine or cell adhesion molecule by vascular endothelial cells and BEC, summarized in Figure 4. First, fractalkine as chemokine from vascular endothelial cells stimulated via TLR3 or TLR4 induce CX3CR1 positive monocytes or NK cells. Second, fractalkine as cell adhesion molecule from TLR4-stimulated BEC recruit CX3CR1 positive cells around target cells. This mechanism may trigger the onset of chronic nonsuppurative destructive cholangitis and autoimmune mechanism perpetuating the cholangitis. We further submit that Th1 chemokines produced by BEC stimulated from TLR3 are important contributors to the autoimmune mechanism.

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＜特別寄稿＞

原発性胆汁性肝硬変（PBC）の診療ガイドライン（2012年）

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付記：本診療ガイドラインは既に「難病情報センター」およびガイドライン専門サイトである「医療情報サービスMinds（マインズ）」のホームページに掲載されているが，日本肝臓学会会員にも広く認識していただくために雑誌「肝臓」に特別掲載されるものである．

はじめに

原発性胆汁性肝硬変（Primary biliary cirrhosis：PBC）は病因が未だ解明されていない慢性進行性の胆汁うっ滞性肝疾患である．1851年にAddison & Gullによって初めて記載され，1950年Ahrensらによって"Primary biliary cirrhosis"と命名された．病理組織学的に慢性非化膿性破壊性胆管炎(chronic non-suppurative destructive cholangitis：CNSDC)と肉芽腫の形成を特徴とし，胆管上皮細胞の変性・壊死によって小葉間胆管が破壊・消滅することにより慢性進行性に胆汁うっ滞を呈する．胆汁うっ滞に伴い肝実質細胞の破壊と線維化を生じ，究極的には肝硬変から肝不全を呈する．臨床的には胆汁うっ滞に伴う瘙痒感，および自己抗体の一つである抗ミトコンドリア抗体(Anti-mitochondrial antibody：AMA）の陽性化を特徴とし，中年以後の女性に多い．臨床症状も全くみられない無症候性PBCの症例も多く，このような症例は長年無症状で経過し予後もよい．本症は種々の免疫異常とともに自己抗体の一つであるAMAが特異的かつ高率に陽性化し，また，慢性甲状腺炎，シェーグレン症候群等の自己免疫性疾患や膠原病を合併しやすいことから，病態形成には自己免疫学的機序が考えられている．しかし，発症の契機となるものは何か，組織障害の機序は何であるのか，現在なお明らかにされていない．

治療薬として，いまだ完全寛解をもたらす薬物の開発はみられないが，ウルソデオキシコール酸(ursodeoxycholic acid；UDCA)が1980年代後半から使用し始められ，現在では第一選択薬となっている．PBCは稀少疾患であり，UDCAを対象とした臨床試験以外はエビデンスレベルが高いランダム化比較試験は多くは行われていない．UDCAのPBC治療への適応開始前と後では，PBC患者の予後も大きく変わった．2009年には，それを踏まえたPBC，あるいは慢性胆汁うっ滞性肝疾患の診療ガイドラインが，それぞれ米国肝臓学会AASLD，ヨーロッパ肝臓学会EASLより発表された．

本診療ガイドラインでは，我が国の実情も踏まえ，我が国の一般内科医，消化器・肝臓医，肝臓専門医がPBC患者の診療にあたって参考にすべき指針をまとめたものである．

2011年3月

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