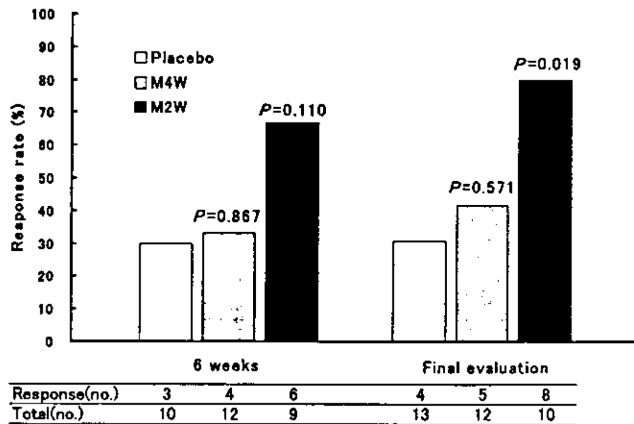

Figure 2. Percentages of patients with Crohn's disease with response (decrease in CDAI score from baseline ≥70 points) according to each treatment group. All significant differences are indicated in the Figure (χ^2 test vs. placebo). *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly.

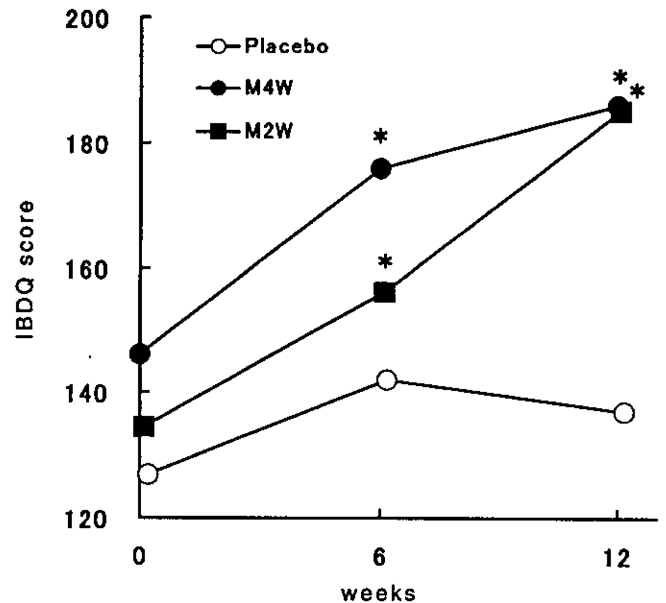

Figure 3. Median IBDQ scores according to each treatment group. *M2W*: MRA 8 mg/kg biweekly; *M4W*: MRA 8 mg/kg/placebo alternately, biweekly; *Placebo*: biweekly. **P* < 0.05: significantly different from baseline based on paired *t* test.

diet at baseline. Only 1 patient in the M4W group was treated with 60 mg/day of azathioprine; none was treated with mercaptopurine. All treatment groups had a mean CDAI score of approximately 300, despite concomitant medications.

Eleven patients discontinued treatment (6 in placebo, 4 in M4W, and 1 in M2W). The reasons for discontinuation were lack of efficacy or withdrawal of consent (5 in placebo and 3 in M4W) and adverse events (1 each in placebo and M4W). One patient in the M4W group was not assessed for CDAI at 2 weeks because of discontinuation owing to a serious adverse event. One patient in the M2W group was reported positive for anti-MRA antibody at baseline and was discontinued according to the protocol, although it was subsequently determined to be a false positive based on a reassessment of the assay method.

Clinical Response to Treatment

With respect to the primary end point, 80% of the patients in the M2W group had a clinical response at the final evaluation that was statistically significantly higher than 31% of the placebo group (Figure 2). Twenty percent of the patients (2 of 10) on this regimen went into remission, as compared with 0% (0 of 13) of the placebo group ($P = 0.092$). The clinical response rate in the M4W group was 42%. The remission rate in this group (25%; 3 of 12; $P = 0.055$ vs. placebo) was similar to the M2W group.

The mean reduction in the CDAI score in the M2W group was 88 points, from 306 (range, 250–384) to 218 (range, 123–334) points; that in the M4W group was 75 points, from 287 (range, 195–393) to 216 (range, 59–463) points; and that in the placebo group was 41 points, from 295 (range, 183–400) to 254 (range, 164–371).

The quality of life measured by the IBDQ improved in the MRA group (Figure 3). In particular, the MRA groups showed a significant increase in the mean score from baseline at 6 weeks and 12 weeks.

Endoscopic examination was performed in 11 patients and evaluated by using CDEIS. As shown in Table 2, there was no significant difference among the groups. Tissue samples were examined for histology in some patients; however, there was no remarkable improvement either. Patients were on stable doses of corticosteroids

Table 2. Changes in CDEIS From Baseline to Final Evaluation

Group	Patient	Baseline	Final evaluation
Placebo[a]	1	13.6	19.1
	2	13.8	14.0
	3	15.5	24.6
	4	10.6	10.5
M4W[b]	1	19.0	21.7
	2	11.1	20.3
	3	12.2	11.6
	4	5.5	4.6
M2W[c]	1	20.9	18.9
	2	9.7	16.4
	3	19.4	14.6

CDEIS, Crohn's disease endoscopic index of severity.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/placebo alternately, biweekly.
[c]MRA, 8 mg/kg biweekly.

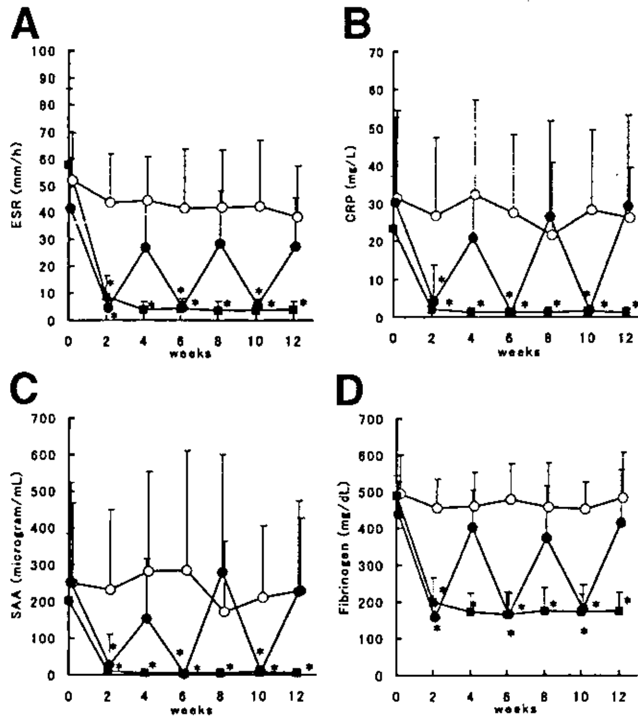

Figure 4. Mean values for (*A*) ESR, (*B*) CRP, (*C*) SAA, and (*D*) fibrinogen concentrations after repeated administration according to each treatment group. ○: Placebo biweekly; ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *$P < 0.05$: significantly different from placebo based on Student *t* test. *Bars* indicate SD.

during the study, and steroid-sparing effect was not investigated in this preliminary study.

Inflammatory Markers

The levels of inflammatory markers including ESR, CRP, SAA, and fibrinogen normalized within 2 weeks after a single dose of MRA (Figure 4). The M2W group, but not the M4W group, maintained the normal levels during the trial period. Increased platelet counts seen at baseline decreased to a normal range at 12 weeks: from 375 to 289 $\times 10^3/mm^2$ in the M2W group, from 378 to 378 $\times 10^3/mm^2$ in the placebo group, and from 324 to 313 $\times 10^3/mm^2$ in the M4W group.

Tolerability and Adverse Events

The infusion was generally well tolerated. As presented in Table 3, the adverse events observed in at least 20% of the patients in any of the groups throughout the study were common cold, nausea, pharyngolaryngeal pain, headache, retching, vomiting, and insomnia. Overall, 5 serious adverse events (SAE), which required hospitalization, were reported: 1 in the M2W group, 2 in the M4W group, and 2 in the placebo group. There were 2 SAEs that led to discontinuation from the study. One patient in the M4W group discontinued the treatment because of paralytic ileus, which developed 13 days after the initial infusion. The symptom resolved within 5 days without any intensive treatment. The causal relationship was determined as "possibly" by the investigator. Another patient in the placebo group discontinued treatment because of a suspected intraperitoneal abscess. The remaining 3 SAEs were abdominal pain/gastrointestinal bleeding in the M2W group, gastrointestinal bleeding in the M4W group, and relapse of a perianal abscess in the placebo group. The gastrointestinal bleeding in the M4W group was determined as a "possible" causal relationship. It cannot be explained that the observed paralytic ileus and gastrointestinal bleeding might be related to blockade of IL-6 function. No serious infusion reactions, occurring on any of the infusion days, were reported in any of the treatment groups. No significant trends were observed in the routine laboratory values. No clinically significant abnormalities were found in electrocardiograms, and no deaths occurred during the trial.

Immunologic Results and Pharmacokinetics

No patient developed antinuclear or anti-DNA antibody during the trial period. Specific antibodies to MRA could not be found in the serum from the patients in any of the treatment groups.

Although paired biopsy before and after treatment was performed in only 2 patients, TUNEL-positive, apoptotic mononuclear cells increased in an M2W patient, whereas no remarkable difference was observed in a placebo-treated patient (Figure 5).

The serum concentrations of MRA were detected 2 weeks after every infusion; however, they were no longer detectable values at 4 weeks (Figure 6). Pharmacokinetic analyses revealed that mean half-life of MRA was 113.17

Table 3. Adverse Events

Variable	Placebo[a]	M4W[b]	M2W[c]
Number of patients evaluated	13	13	10
Adverse event, No. (%)			
Common cold	3 (23)	3 (23)	2 (20)
Nausea	2 (15)	3 (23)	2 (20)
Pharyngolaryngeal pain	3 (23)	1 (8)	2 (20)
Headache	2 (15)	1 (8)	2 (20)
Retching	1 (8)	0	3 (30)
Vomiting	1 (8)	1 (8)	2 (20)
Insomnia	1 (8)	0	2 (20)

NOTE. Adverse events that occurred in 20% or more of the patients in any of the groups are reported.
[a]Placebo, biweekly.
[b]MRA, 8 mg/kg/Placebo alternately, biweekly.
[c]MRA 8 mg/kg biweekly.

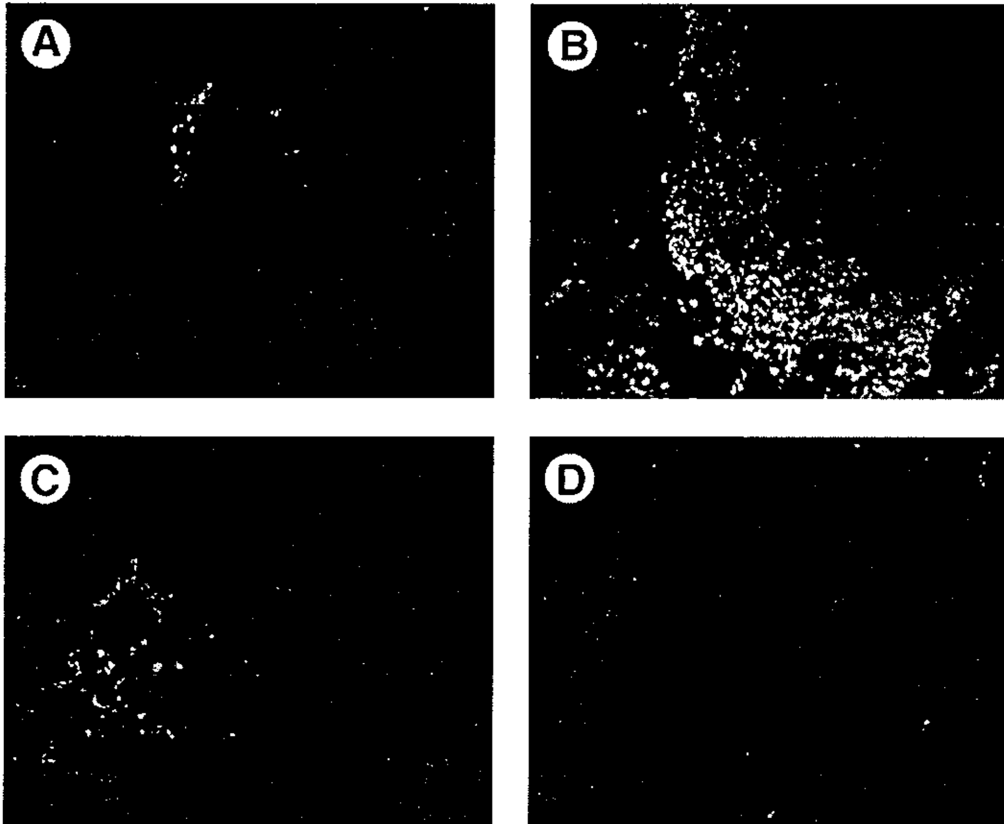

Figure 5. MRA induced apoptosis. Pairs of colonic tissue samples taken from an M2W patient before (*A*) and after treatment (*B*) and from a placebo patient before (*C*) and after treatment (*D*) were stained by using fluorescent TUNEL assay kit (MBL Co., Ltd., Nagoya, Japan; original magnification 100×).

hours in the M2W group and 97.34 hours in the M4W group. The mean volumes of distribution were 63.56 and 64.65 mL/kg in the M2W and M4W groups, respectively. Serum concentration of IL-6 and sIL-6R increased after administration of MRA; however, repetitive infusions of MRA did not induce further increase of these concentrations (Figure 7).

Discussion

Our study is the first randomized placebo-controlled trial of anti-IL-6R mAb MRA in the treatment of patients with active CD. Although this is a preliminary study, the results presented here show that the therapy with MRA for CD is safe and well tolerated and suggests a beneficial effect.

The clinical response rate of the M2W group was higher than that of the M4W group. The different response rate between the MRA groups might be attributable to a continuous suppression of acute-phase reactants such as CRP, ESR, SAA, and fibrinogen for M2W in contrast to M4W, and it is considered that such suppression may require the presence of MRA in the

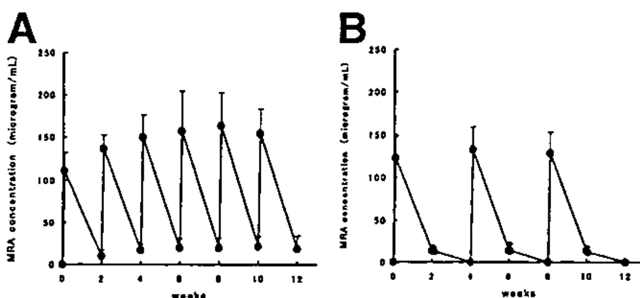

Figure 6. Mean serum MRA concentration (mg/mL) after repeated administration. Serum samples were collected before each infusion and 1 hour after each infusion and at week 12. (*A*) MRA 8 mg/kg were infused biweekly. (*B*) MRA 8 mg/kg or placebo were infused alternately, biweekly. The concentrations were under the limit of detection before each infusion at week 4, 8, and at week 12. *Bars* indicate SD.

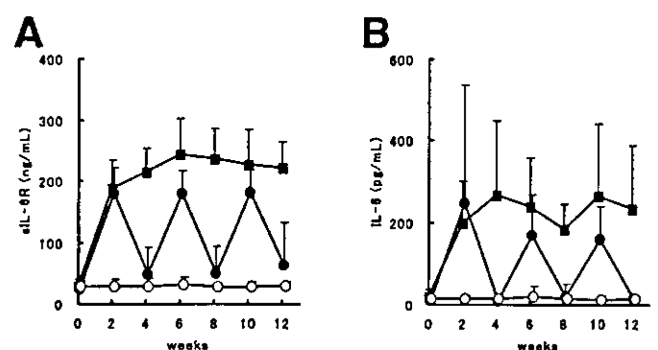

Figure 7. Mean values for (*A*) concentration of IL-6 and (*B*) concentration of sIL-6R after repeated administration according to each treatment group. ○: Placebo biweekly, ●: MRA 8 mg/kg/placebo alternately, biweekly; ■: MRA 8 mg/kg biweekly. *Bars* indicate SD.

serum throughout the treatment period. The levels of acute-phase reactants were completely normalized after only a single dose of MRA. A similar result was observed after administration of MRA for rheumatoid arthritis.[9,13] In studies of other anti-cytokine therapies, such as TNF-α blockers[14–16] and IL-1R antagonist,[17] the acute-phase reactants decreased with the treatment but not to normal levels. Therefore, it is now unquestionable that IL-6 is the principal cytokine responsible for the production of acute-phase reactants in both rheumatoid arthritis and CD. It has been shown that CRP is not a mere serum marker of inflammation but is a promoter of the IL-6R shedding to supply sIL-6R.[18] Therefore, normalization of CRP itself seems to be of benefit in the treatment of CD.

The effect on the anal complications of CD was not included in the principal evaluation of this study; however, 4 out of 6 MRA-treated patients showed disappearance of anal fissure, and 2 out of 10 showed closure of anal fistula, whereas none in the placebo group showed improvement in any of these lesions. Endoscopic and histologic healing was reported after infliximab therapy; however, such healing was not observed in this trial.[19] Although TUNEL staining displayed increased apoptosis of mononuclear cells by MRA treatment, induction of apoptosis was not conclusive because only 1 paired biopsy specimen each from MRA and placebo patient were obtained. Further study is needed to provide definitive results.

It is notable in this pilot study that MRA did not induce autoantibodies or antibodies to MRA itself, although no patients received any immunosuppressive drugs except 1 in the M4W group (azathioprine), in contrast to anti-TNF-α antibody.[14,20] Emergence of autoantibodies such as anti-nuclear antibody and anti-DNA antibody was observed in some patients treated with TNF-α blockers.[20] These antibodies are often seen in patients with systemic lupus, and reduced TNF-α levels were correlated with severe disease in lupus nephritis model,[21] which suggests that TNF-α and IL-6 have different relevance in the autoimmune phenomenon, and blocking the latter might have better safety, although the number of patients was limited in this study. Furthermore, there was no incidence of serious infusion reactions and infections during the treatment period.

This study was performed in Japan, and we have to be circumspect in comparing this study with other therapies because the modalities of the therapy for CD might be different from the Western countries, e.g., the use of steroids, immunosuppressive drugs, and elemental diet. The difference of ethnic background should also be taken into consideration. Therefore, it is desirable that the study of MRA be extended to the countries where other studies were carried out.

In conclusion, this preliminary study shows that a biweekly 8 mg/kg infusion of MRA for 12 weeks is safe and well tolerated by the patients with active CD and showed a significantly higher response rate than placebo, although endoscopic and histologic healing was not observed during the trial period. Striking improvement was observed in the acute-phase reactants, which confirms that IL-6 is the major cytokine responsible for their production in CD. Further work is needed to establish the safety and efficacy of MRA in a larger population of the patients with CD.

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Received April 4, 2003. Accepted January 8, 2004.

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Supported by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.

The authors wish to thank Paul Langman, Ph.D., for his valuable assistance with the preparation of the English version of this paper.

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IRRITABLE BOWEL SYNDROME

T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated immunopathology in Crohn's disease

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Gut 2004;**53**:1303–1308. doi: 10.1136/gut.2003.024190

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Revised version received 29 January 2004
Accepted for publication 3 March 2004

Background and aims: Many lines of evidence suggest that T helper cell type 1 (Th1) immune responses predominate in Crohn's disease (CD). Recently, a novel transcription factor T-box expressed in T cells (T-bet) has been reported as the master regulator of Th1 development. This study was designed to investigate the role of T-bet and proinflammatory cytokines in Th1 mediated immunopathology in CD.
Materials: CD4+ lamina propria mononuclear cells (LPMCs) were isolated from surgically resected specimens (CD, n = 10; ulcerative colitis (UC), n = 10; normal controls (NL), n = 5).
Methods: (1) T-bet expression of CD4+ LPMCs was examined by quantitative real time polymerase chain reaction and western blotting. (2) T-bet expression of LPMCs stimulated by interleukin (IL)-12/IL-18 was analysed by western blotting. (3) Interferon γ (IFN-γ) production and T-bet expression of CD4+ peripheral blood mononuclear cells (PBMCs) were examined with or without stimulation by anti-CD3/CD28 monoclonal antibodies and/or IL-12.
Results: (1) T-bet expression of CD4+ LPMCs was increased in CD compared with UC and NL. (2) Synergistically, augmentation of IFN-γ production by IL-12/IL-18 was independent of T-bet expression in LPMCs. (3) T-bet was induced by T cell receptor stimulation in CD4+ PBMCs. T-bet induction correlated with IFN-γ production and with augmentation of surface expressed IL-12 receptor β2.
Conclusions: T-bet induction by antigenic stimulation and subsequent stimulation by macrophage derived IL-12/IL-18 are important for establishing Th1 mediated immunopathology in CD.

Crohn's disease (CD) is a chronic inflammatory process involving the gastrointestinal tract, characterised by discontinuous and transmural inflammation. Although the aetiology of CD is not fully understood, accumulating evidence suggests that dysregulation of the local immune system is pivotal in the pathogenesis of CD.[1 2] Studies from humans and experimental murine colitis models indicate that in CD, the local immune response tends to be predominantly T helper cell type 1 (Th1) and is reflected by local release of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-12, and IL-18.[3–7] Studies conducted in experimental murine colitis models showing that neutralising antibodies against TNF-α, IL-12, or IL-18 prevent the onset of colitis lend further support to a Th1 predominant immunopathology in CD.[8–10] Indeed, antihuman TNF-α monoclonal antibody (mAb) (Infliximab) is effective for many patients with CD refractory to conventional therapy.[11 12] Collectively, these observations indicate that Th1 mediated immunopathology plays a central role in induction and perpetuation of intestinal inflammation in CD.

Given the observed polarised nature of T cells in CD, understanding the mechanisms that lead to the establishment of this polarised state in the gastrointestinal mucosa is critical. Naïve CD4 T lymphocytes in transit to becoming either Th1 or Th2 effector cells undergo sequential stages of cytokine activation, commitment, silencing, and physical stabilisation during polarisation into differentiated effector subsets, a process tightly controlled by regulatory transcription factors.[13 14] Various transcription factors such as c-maf, GATA-3, and STAT-6 have been shown to promote expression of several Th2 cytokines, including IL-4, IL-5, and IL-13, either by transactivation of cytokine gene promoters and enhancers or induction of chromatin remodelling.[15–17] In contrast with Th2 differentiation, very little is known about the molecular basis of Th1 differentiation. STAT-4 and IRF-1 are specifically associated with IL-12 and interferon (IFN)-γ signalling in T cells, respectively, and play a key role in regulating cytokine production of Th1 cells at the transcriptional level.[13 18]

Recently, T box expressed in T cells (T-bet), a member of the T-box family of transcription factors, has been shown to transactivate the *IFN-γ* gene.[19] T-bet, whose expression is primarily limited to the immune system, is rapidly induced in early developing Th1 cells and is absent in developing Th2 cells.[19] T-bet deficient mice show normal lymphoid development but exhibit marked impairment in mounting Th1 mediated immune responses in response to IL-12.[20] Moreover, retroviral mediated expression of T-bet in Th2 cells leads to induction of a Th1 cytokine profile in these cells.[19] Thus T-bet initiates Th1 cell differentiation by activating Th1 genetic programmes and repressing Th2 programmes.

Neurath *et al* have demonstrated that expression of T-bet is increased in CD and that T-bet regulates the mucosal cytokine balance in various murine experimental colitis models.[21] However, the pathophysiological role of T-bet in human CD has not been fully described. In the present study,

Abbreviations: CD, Crohn's disease; UC, ulcerative colitis; NL, normal control; IL, interleukin; IL-12Rβ2, IL-12 receptor β2; LPMC, lamina propria mononuclear cell; PBMC, peripheral blood mononuclear cell; T-bet, T-box expressed in T cells; TCR, T cell receptor; Th1, T helper cell type 1; IFN-γ, interferon γ; mAb, monoclonal antibody; TNF-α, tumour necrosis factor α; PCR, polymerase chain reaction; AU, arbitrary units; TGF-β, transforming growth factor β

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we investigated the role of T-bet in induction of Th1 responses in human CD, and differences in immune responses between CD and ulcerative colitis (UC).

MATERIALS AND METHODS

Patients and samples

Mucosal samples were obtained from surgically resected inflamed areas of intestinal specimens from 10 patients with CD and 10 with UC. Patient profiles are summarised in table 1. CD or UC was diagnosed based on clinical, radiographic, endoscopic, and histological findings by established criteria.[22 23] The degree of inflammation was histologically moderate to severe in all samples. Normal controls (NL) included mucosal samples from macroscopically and microscopically unaffected areas from patients with sporadic colon cancer. All experiments were approved by the local ethics committees. Informed consent was obtained from all patients before obtaining the samples.

RNA extraction and quantitative real time polymerase chain reaction (PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was treated with Qiagen DNase (Qiagen) to remove any contaminating genomic DNA. Complementary DNA (cDNA) was synthesised using the Superscript first strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Quantitative real time PCR was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, California, USA) with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). We confirmed that non-specific bands were not detected by melting curve analysis for each primer set. T-bet mRNA transcripts were normalised with β-actin mRNA transcripts and expressed as arbitrary units (AU). PCR primers were as follows: T-bet forward, 5'-CCC CCA AGG AAT TGA CAG TTG-3'; reverse 5'-GGG AAA CTA AAG CTC ACA AAC-3'. β-Actin forward, 5'-AAG CAG GAG TAT GAC GAG TCC G-3'; reverse, 5'-CGG AAC TAA GTC ATA GTC CGC C-3'

Preparation of lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs)

LPMCs were isolated from surgically resected intestinal specimens using enzymatic techniques, as previously described.[3] Briefly, dissected mucosa was incubated in calcium and magnesium free Hanks' balanced salt solution (Sigma, St Louis, Missouri, USA) containing 2.5% fetal bovine serum (BioSource, Camarillo, California, USA) and 1 mM dithiothreitol (Sigma). The mucosa was then incubated in medium containing 0.75 mM EDTA (Sigma) for 60 minutes at 37°C. During this treatment, intraepithelial lymphocytes and epithelial cells were removed from the tissue. Then, tissues that contained LPMCs were collected and incubated in medium containing 0.02% collagenase (Worthington Biochemical Corp, Freehold, New Jersey, USA). The fraction was pelleted and cells centrifuged over a 40–60% Percoll solution (Amersham Biosciences Corp, Piscataway, New Jersey, USA) density gradient. PBMCs were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway) from heparinised peripheral blood samples. LPMCs or PBMCs were further separated into CD4 positive cells using MACS (Miltenyi Biotec, Auburn, California, USA) according to the manufacturer's instructions.

Table 1 Clinical profiles of Crohn's disease (CD) and ulcerative colitis (UC) patients

	CD	UC
No of patients	10	10
Sex (F/M)	4/6	6/4
Age (y) (mean (range))	33.0 (21–51)	39.4 (22–70)
Disease activity*		
CDAI (mean (range))	224.8 (165–324)	–
CAI (mean (range))	–	9.2 (5–16)
Medication		
5-ASA or SASP	9	8
Steroid	2	3
Azathioprine	3	1

*Assessment of disease activity using Crohn's disease activity index (CDAI) in CD and clinical activity index (CAI) in UC.
5-ASA, 5-aminosalicylic acid, SASP, salicylazosulphapyridine.

Cell culture

LPMCs and CD4+ PBMCs were cultured at a concentration of 1×10^6/ml in complete RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). For CD4+ PBMCs culture, 10 μg/ml of immobilised anti-CD3 (OKT3) and 5 μg/ml of anti-CD28 (CD28.2; BD Pharmingen, San Diego, California, USA) antibodies were used. Recombinant IL-12p70 (BD Pharmingen) and/or IL-18 (MBL, Nagoya, Japan) were added in culture medium, as indicated.

Enzyme linked immunosorbent assay (ELISA)

Concentrations of IFN-γ and IL-12 in culture supernatants of LPMCs and PBMCs were measured using specific ELISA (IFN-γ: Endogen, Woburn, Massachusetts, USA, IL-12: BioSource, Camarillo, California, USA). According to the manufacturer's instructions, the minimum detectable IL-12 and IFN-γ concentrations were 7.8 pg/ml and 25.6 pg/ml, respectively.

Flow cytometry

Flow cytometric analysis was performed as previously described.[9] Phycoerythrin conjugated antihuman IL-12 receptor β2 (IL-12Rβ2) antibody was purchased from BD Pharmingen. Fluorescence intensity on the surface of the cells was analysed using a FACScan (Becton Dickinson, Mountain View, California, USA).

Protein extraction and western blotting

Total protein was extracted using lysis buffer containing 10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and a mixture of protease inhibitors. Total protein (10 μg) was separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and electrophoretically transferred onto Immobilon-P membrane (Millipore, Bedford, Massachusetts, USA). To detect T-bet protein, the membrane was incubated with rabbit anti-T-bet antisera (1:3000 final dilution; kindly provided by Drs L Glimcher and S Szabo, Harvard, School of Public Health, Boston, Massachusetts, USA) and subsequently with horseradish peroxidase conjugated goat antirabbit IgG Ab (1:2000, New England Biolabs, Beverly, Massachusetts, USA). Antibody reactions were detected with a chemiluminescence detection kit (Amersham Biosciences Corp). The membrane was subsequently stripped with Restore western blot stripping buffer (Pierce, Rockford, Illinois, USA) and incubated with mouse anti-β-actin Ab (1:2000, Sigma). Densitometric analysis was performed with NIH image software version 1.61 and T-bet expression was adjusted to β-actin expression.

Statistical analysis

Statistical differences were analysed using the Mann-Whitney U test. A p value of <0.05 was considered to be significant. All data are expressed as mean (SEM).

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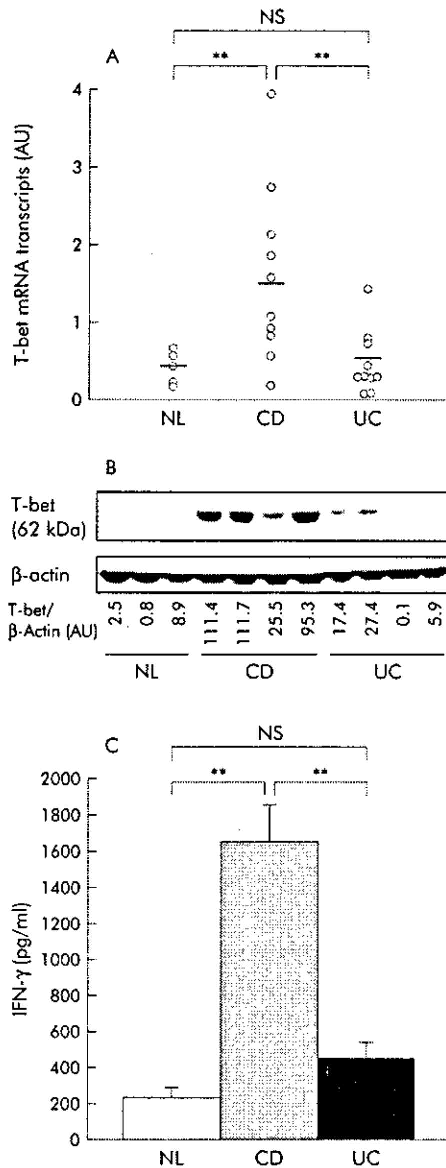

Figure 1 T-box expressed in T cells (T-bet) expression of CD4+ lamina propria mononuclear cells (LPMCs) was increased in Crohn's disease (CD). Total RNA was extracted from CD4+ LPMCs (CD, n = 10; ulcerative colitis (UC), n = 10; normal controls (NL), n = 5). (A) Levels of T-bet mRNA transcripts were measured by quantitative real time polymerase chain reaction and adjusted to β-actin mRNA transcripts. **p<0.01. (B) T-bet protein expression was assessed by western blotting in CD and UC patients and in NL. AU, arbitrary units. (C) LPMCs were cultured in medium alone for 48 hours (CD, n = 6; UC, n = 6; NL, n = 6). Production of interferon γ (IFN-γ) was measured by ELISA. **p<0.01.

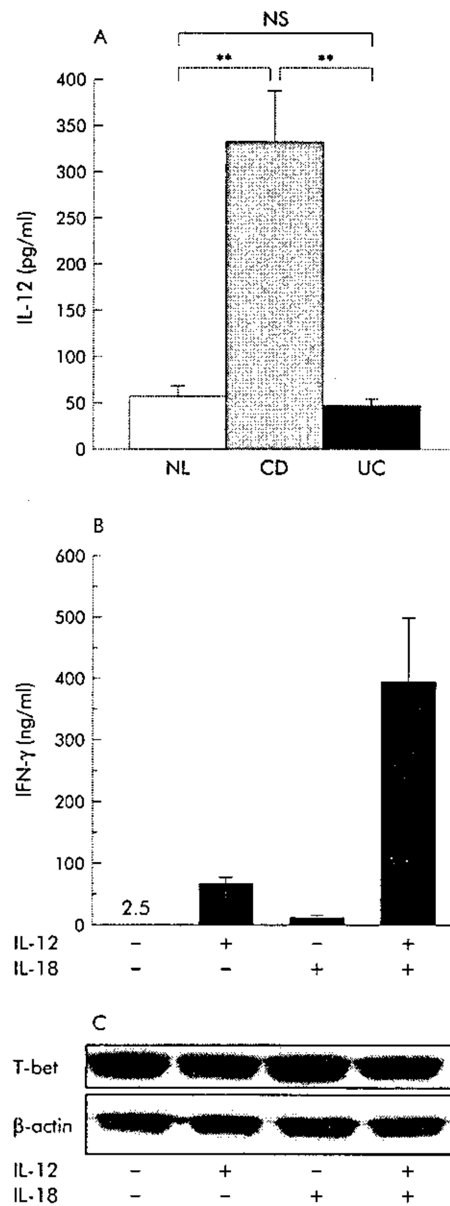

Figure 2 T-box expressed in T cells (T-bet) did not contribute to synergistic augmentation of interferon γ (IFN-γ) production by interleukin (IL)-12 and IL-18 stimulation. (A) Lamina propria mononuclear cell (LPMCs) were cultured in medium alone for 48 hours. Production of IL-12 was measured by ELISA in Crohn's disease (CD) and ulcerative colitis (UC) patients, and in normal controls (NL). **p<0.01. (B) LPMCs from CD patients were stimulated by IL-12 (1 ng/ml) and/or IL-18 (1 ng/ml) for 48 hours. Production of IFN-γ was measured by ELISA (n = 6). (C) T-bet protein expression was assessed by western blotting. Results are representative of three independent experiments.

RESULTS

T-bet was upregulated in CD4+ LPMCs of CD

To clarify the involvement of T-bet in Th1 mediated immunopathology in CD, we first assessed expression of T-bet mRNA in CD4+ LPMCs obtained from five NL, 10 CD patients, and 10 UC patients using quantitative real time PCR. T-bet mRNA obtained from CD4+ LPMCs of CD patients (1.59 (0.38) AU) was significantly ($p<0.01$) increased compared with that obtained from UC patients (0.48 (0.14) AU) and NL (0.41 (0.11) AU) (fig 1A). T-bet protein was also significantly ($p<0.05$) increased in CD4+ LPMCs of CD patients (88.5 (21.3) AU) compared with those of UC patients (12.7 (7.2) AU) and NL (4.1 (3.1) AU) (fig 1B). T-bet expression was not significantly different between UC and NL at both the mRNA and protein levels.

To assess the correlation between T-bet expression and Th1 responses, we next examined IFN-γ production by LPMCs cultured without any stimuli for 48 hours, as measured by ELISA. As shown in fig 1C, IFN-γ production by LPMCs from patients with CD was significantly ($p<0.01$) higher (1659.0 (206.7) pg/ml) than that from patients with UC (461.5 (88.1) pg/ml) or from NL (233.3 (61.4) pg/ml). This enhanced production of IFN-γ well corresponded to augmentation of

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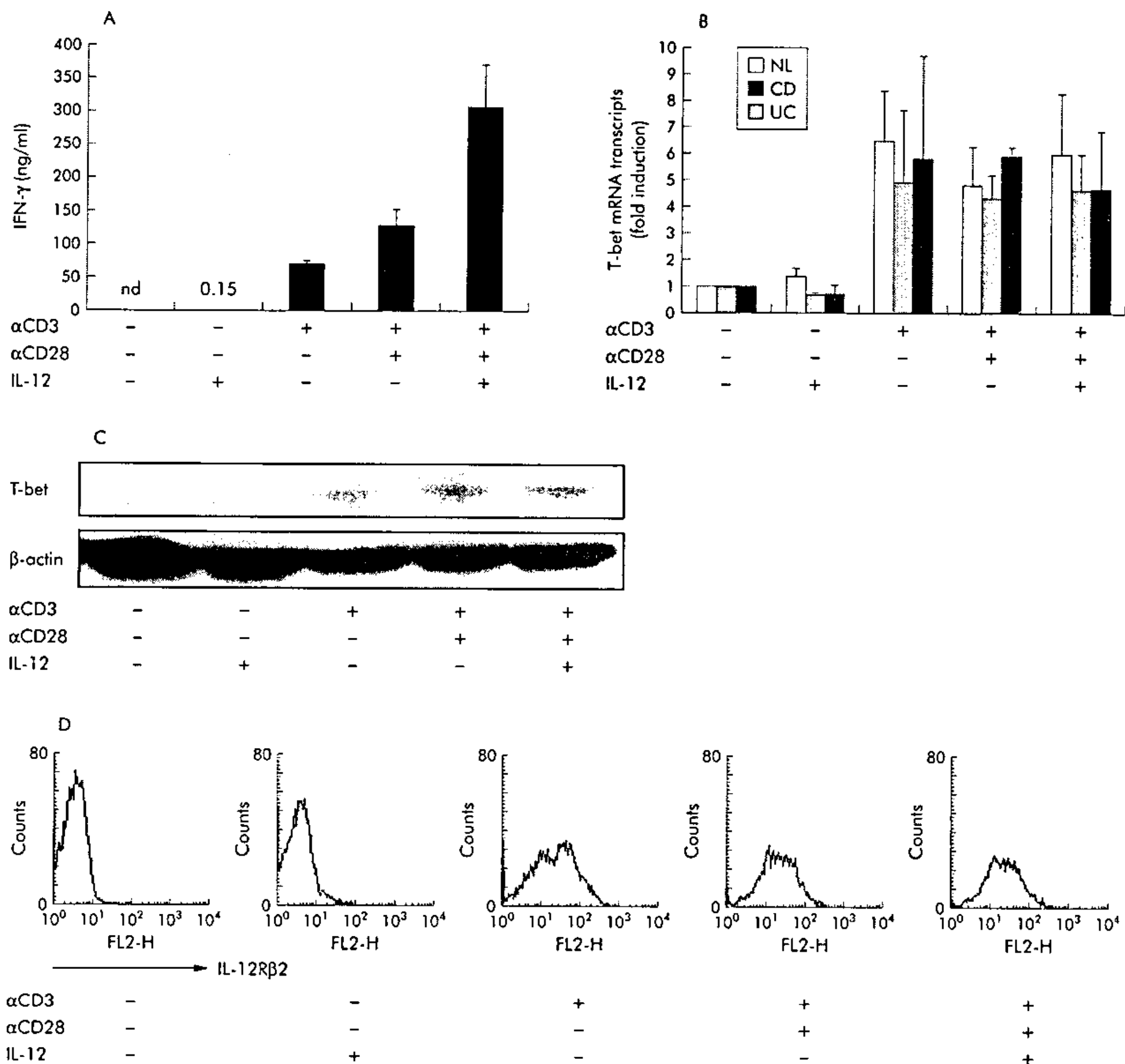

Figure 3 Induction of T-box expressed in T cells (T-bet) via the T cell receptor (TCR) signalling pathway is necessary for interferon γ (IFN-γ) production before interleukin (IL)-12 stimulation. CD4+ PBMCs were stimulated with anti-CD3 (10 μg/ml)/CD28 (5 μg/ml) and/or IL-12 (10 ng/ml) for 48 hours. (A) Production of IFN-γ was measured by ELISA (n = 3). (B) T-bet mRNA expression was analysed by quantitative real time polymerase chain reaction after stimulation for 12 hours in patients with Crohn's disease (CD) and ulcerative colitis (UC), and in normal controls (NL). (C) T-bet protein expression was assessed by western blotting. (D) Surface expression of IL-12Rβ2 was analysed by FACS.

T-bet expression by LPMCs from patients with CD. These results indicated that T-bet may essentially contribute to Th1 immune responses in CD.

T-bet did not contribute to synergistic augmentation of IFN-γ production by IL-12 and IL-18 stimulation

To investigate which stimuli induce T-bet in CD, we focused on Th1 inducing cytokines such as IL-12, which is secreted by activated macrophages and dendritic cells via activation of innate immune responses. We first measured spontaneous IL-12 production from LPMCs. As previously reported,[24] IL-12 production by LPMCs from patients with CD (333.3 (54.7) pg/ml) was significantly ($p<0.01$) higher than that from patients with UC (48.4 (7.1) pg/ml) or NL (56.7 (11.4) pg/ml) (fig 2A). We next examined whether IL-12 and/or another Th1 inducing cytokine IL-18 could regulate T-bet expression in LPMCs from patients with CD. While IL-12 or IL-18 by themselves increased IFN-γ production by LPMCs from patients with CD, in combination a dramatic effect on IFN-γ production by cells was noted. However, neither single nor combined addition of IL-12/IL-18 significantly upregulated T-bet expression (fig 2C). These results demonstrate that synergistic augmentation of IFN-γ production by IL-12 and IL-18 in CD patients did not require further T-bet upregulation, suggesting a T-bet independent pathway.

Induction of T-bet via the T cell receptor (TCR) signalling pathway is necessary for IFN-γ production before IL-12 stimulation

Next, we hypothesised that TCR stimulation was important for T-bet induction early in Th1 mediated immunopathology in CD. Because LPMCs are reported to show activated phenotypes,[2] indicating that they have already experienced antigenic stimulation in the intestinal mucosa, we used CD4+

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PBMCs in the following experiments. CD4+ PBMCs were stimulated with plate bound anti-CD3 and/or CD28 mAb in the presence or absence of IL-12. After in vitro activation, IFN-γ production and T-bet expression were analysed. As shown in fig 3A, CD4+ PBMCs from CD patients produced little IFN-γ in the presence of IL-12 without plate bound anti-CD3 mAb stimulation. Under these conditions, T-bet was not augmented at either the mRNA or protein level (fig 3B, C). In contrast, anti-CD3 mAb stimulation without exogenous IL-12 induced a large amount of IFN-γ (68.5 (5.0) ng/ml), as well as marked expression of T-bet (fig 3A–C). The level of T-bet induction by anti-CD3 mAb stimulation was not significantly different among CD4+ PBMCs from CD or UC patients, or NL (fig 3B). Consistent with mRNA expression, T-bet protein was augmented by anti-CD3 mAb stimulation in CD4+ PBMCs from patients with CD (fig 3C; similarly in UC and NL, but data not shown). Furthermore, in parallel with T-bet induction by anti-CD3 mAb stimulation, IL-12Rβ2 was also induced in CD4+ PBMCs (fig 3D) and additional stimulation by IL-12 induced a fivefold greater amount of IFN-γ (355.0 (62.2) ng/ml) than anti-CD3 mAb stimulation alone (fig 3A). However, IL-12 costimulation did not induce further T-bet upregulation (fig 3B, C) which was consistent with data regarding LPMCs shown in fig 2C. It is reported that transcription of IL-12Rβ2 was regulated by T-bet in mice.[25 26] Collectively, these results indicate that T-bet is induced through TCR stimulation prior to IL-12 signalling for enhanced IFN-γ production; T-bet may not only initiate IFN-γ production but also control the responsiveness to IL-12 through upregulation of IL-12Rβ2.

DISCUSSION

Although CD represents typical Th1 mediated immune responses, the molecular mechanism involved in differentiation of Th1 cells in CD has not been elucidated. T-bet has been proposed to be the master regulator of Th1 development based on its induction of IFN-γ and repression of Th2 cytokines.[19] Furthermore, a study in mice showed that T-bet was STAT-4 independent and acted before IL-12 in Th1 development.[25] These observations led us to consider the role of T-bet in human inflammatory bowel diseases, especially in CD. Here we demonstrated that T-bet was expressed strongly in CD4+ LPMCs from CD patients compared with those from UC patients and NL. Neurath *et al* demonstrated that T-bet was strongly expressed in CD3+ LPMCs in patients with CD while only weak expression was observed in NL and patients with UC.[21] A definitive conclusion could not be drawn from their results because CD3+ T lymphocytes included both CD4+ and CD8+ T lymphocytes, and T-bet is critical for IFN-γ production in CD4+ but not in CD8+ T lymphocytes.[20] Accordingly, we focused on isolated CD4+ LPMCs to study the role of T-bet in induction of Th1 immune responses in CD. In this study, we showed upregulation of T-bet in CD4+ LPMCs from CD. Increased expression of T-bet in CD was consistent with enhanced production of IFN-γ from LPMCs of CD. Collectively, these results indicate that T-bet may play an essential role in induction of Th1 immune responses in CD.

What regulates T-bet augmentation in CD? We demonstrated that induction of T-bet in human CD4+ PBMCs was mediated by anti-CD3 mAb, but not IL-12, suggesting that antigenic stimulation was essential for T-bet induction. Although some studies have suggested that IFN-γ/STAT-1 signalling is the key pathway for T-bet expression,[26 27] another study revealed that TCR signalling could maintain T-bet expression in committed CD4+ T cells.[28] The result indicated that repeated antigenic stimulations were essential for retaining high T-bet expression. It is well known that the gastrointestinal tract is continuously exposed to a large amount of antigens derived from the diet, bacteria, and pathogens. Sustained dysregulated T cell responses to ubiquitous antigens could result in continuous upregulation of T-bet in CD. Hyporesponsiveness (tolerance) against various luminal antigens in normal intestinal mucosa is maintained through anti-inflammatory cytokines such as transforming growth factor-β (TGF-β). It was reported that TGF-β suppressed T-bet expression.[21 29] Considering that mucosal T cells from CD patients have been shown to be insensitive to TGF-β inhibition,[30] disruption of TGF-β signalling may possibly be involved in overexpression of T-bet as well as hyperreactivity against various luminal antigens. These findings are consistent with the finding that elimination of luminal antigens by elemental diet or decontamination of gut flora can attenuate mucosal inflammation in human CD and murine experimental colitis models.[31–34]

Although induction of T-bet by anti-CD3 mAb in CD4+ PBMCs was not different between CD and UC, increased expression of T-bet was observed only in CD4+ LPMCs from patients with CD. These data suggest that the cytokine environment in UC is different from that in CD (that is, increased production of IL-5, which is a typical Th2 cytokine).[5] It was reported that Th2 skewing conditions suppressed T-bet expression.[19] Therefore, in vivo T-bet expression in the presence of chronic gut inflammation may be influenced by the local environment, including cytokine patterns. Further studies would however be necessary to clarify these points.

What then is the role of T-bet in induction of Th1 mediated immunopathology in CD? In the present study, we demonstrated that IL-12 in itself could not induce IFN-γ production from CD4+ PBMCs that did not express T-bet. In contrast, anti-CD3 mAb stimulation induced T-bet expression in CD4+ PBMCs and caused them to produce IFN-γ. Activated CD4+ PBMCs also increased IL-12Rβ2 and responded to IL-12 for further production of IFN-γ. It has been reported that transcription of IL-12Rβ2 is regulated by T-bet in mice.[25 26] We and other investigators have previously demonstrated that LPMCs from CD patients expressed high levels of IL-12Rβ2 and responded to IL-12 more strongly than those from NL.[35 36] Consistent with previous reports, our data indicate that T-bet is necessary for initiating IFN-γ production, and that it regulates IL-12 responsiveness through IL-12Rβ2 upregulation in CD.

We have demonstrated here that T-bet expression was increased in CD4+ LPMCs from patients with CD. T-bet induced by antigens in the gut lumen initiates IFN-γ production and modulates IL-12 responsiveness through upregulation of IL-12Rβ2. Furthermore, dysregulated production of IL-12/IL-18, as observed specifically in CD patients in our previous and present studies, may intensify Th1 polarisation of the majority of T cells initiated by T-bet. Thus serial actions by antigenic stimulation to induce T-bet in LPMCs and macrophage derived IL-12/18 could be essential for establishing Th1 mediated immunopathology in CD.

ACKNOWLEDGEMENTS

We thank Professor Daniel Podolsky and Dr Cosmas Giallourakis for critical comments, Drs L Glimcher and S Szabo for providing the antibodies, Professor M Kitajima and Drs M Watanabe, H Hasegawa, Y Iwao, and M Naganuma for providing the specimens, and Dr H Takaishi for helpful discussion. This work was supported in part by grants-in-aid from the Japanese Ministry of Education, Culture, and Science, the Japanese Ministry of Labor, Health, and Welfare, Japan Health Sciences Foundation, Keio University, and Keio University Medical Fund.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1302

The peripheral blood smear revealed numerous deformed red blood cells with thorny projections, consistent with spur cells. The increased free cholesterol/phospholipid molar ratio in the erythrocyte membrane was also noted. Magnetic resonance imaging demonstrated diffuse hypointensity of the liver, suggesting heavy iron loading. Spur cell anaemia of alcoholic cirrhosis was diagnosed.

Spur cell anaemia (acanthocytosis), a rare acquired haemolytic anaemia observed mainly in the end stages of alcoholic cirrhosis, is characterised by an increased ratio of free cholesterol to phospholipid in the erythrocyte membranes that results in multispiculated erythrocytes (acanthocytes). These acanthocytes undergo rapid splenic destruction and consequently have a shortened survival. Recent studies have indicated that alcoholic iron overload may be associated with spur cell anaemia rather than hereditary haemochromatosis. Patients usually need frequent blood transfusions and the prognosis is extremely poor. Liver transplantation, which improves hepatic function and resolves spur cell anaemia, has been the most effective treatment. Our patient did not choose liver transplantation. She had a poor response to conservative treatment with multiple blood transfusions and died of liver failure seven months later.

doi: 10.1136/gut.2003.034785

Cytolytic activity and regulatory functions of inhibitory NK cell receptor–expressing T cells expanded from granulocyte colony-stimulating factor–mobilized peripheral blood mononuclear cells

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Inhibitory natural killer cell receptor (NKR)–expressing cells may induce a graft-versus-leukemia/tumor (GVL/T) effect against leukemic cells and tumor cells that have mismatched or decreased expression of HLA class I molecules and may not cause graft-versus-host disease (GVHD) against host cells that have normal expression of HLA class I molecules. In our study, we were able to expand inhibitory NKR (CD94/NKG2A)–expressing CD8+ T cells from granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cells (G-PBMCs) by more than 500-fold using stimulation by an anti-CD3 monoclonal antibody with interleukin 15 (IL-15). These expanded and purified CD94-expressing cells attacked various malignant cell lines, including solid cancer cell lines, as well as the patients' leukemic cells but not autologous and allogeneic phytohemagglutinin (PHA) blasts in vitro. Also, these CD94-expressing cells prevented the growth of K562 leukemic cells and CW2 colon cancer cells in NOD/SCID mice in vivo. On the other hand, the CD94-expressing cells have low responsiveness to alloantigen in mixed lymphocyte culture (MLC) and have high transforming growth factor (TGF)–β1– but low IL-2–producing capacity. Therefore, CD94-expressing cells with cytolytic activity against the recipient's leukemic and tumor cells without enhancement of alloresponse might be able to be expanded from donor G-PBMCs. (Blood. 2004;104:768-774)

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Introduction

The regulation of graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) effect is the most important issue in allogeneic stem cell transplantation (allo SCT).

It recently has been shown that inhibitory natural killer cell receptors (NKRs) on NK cells negatively regulate NK cell functions through their binding to major histocompatibility complex (MHC) class I molecules.[1-3] It also has been revealed that T cells, especially memory CD8+ T cells, expressed NKRs. NK-like activity and T-cell receptor (TCR)–mediated killing activity of cytotoxic T lymphocytes (CTLs) expressing inhibitory NKRs were suppressed by HLA class I recognition by the NKRs. Inhibitory NKR-positive cells could attack class I–negative target cells but not the same class I–positive cells.[4-7]

Partially HLA-matched bone marrow transplantation (BMT) resulted in a large expansion of donor-derived CTLs expressing CD158b inhibitory NKRs, which did not cause GVHD but allowed a discriminatory GVL reaction.[8] Also, based on the rule of NKR incompatibility, the GVL effectors may be operational in patients who have undergone HLA-mismatched hematopoietic cell transplantation.[9] Mixed lymphocyte reaction and anti-CD3 mAb–redirected cytotoxicity were inhibited by engagement of transgenic CD158b molecules in CD158b transgenic mice.[10,11] Ruggeri et al[12] reported surprisingly good clinical results that indicated no relapse, no rejection, and no acute GVHD after HLA-haplotype–mismatched transplantations with NKR ligand incompatibility in the GVH direction for acute myeloid leukemia (AML) patients. They also reported that donor allogeneic NK cells attacked host antigen-presenting cells (APCs), resulting in the suppression of GVHD. With regard to the clinical advantage of NKR ligand incompatibility in allo SCT from an unrelated donor, Davies et al[13] showed negative data without using antithymocyte globulin (ATG), while Giebel et al[14] showed positive data using ATG as part of GVHD prophylaxis.

In our previous studies, the proportion of CD158b, which is a specific receptor for HLA-C, on CD8+ T cells was found to be increased in patients with chronic GVHD (cGVHD). Also, the proportion of CD94/NKG2A, which is a specific receptor for HLA-E,[15] on T cells was larger in cGVHD patients with good prognosis than in cGVHD patients with poor prognosis. Furthermore, the addition of CD94-enriched fractions to CD94-depleted fractions suppressed the proliferation of T cells in MLCs.[16-19] Therefore, NKR-expressing cells might be involved in the regulation of allogeneic response after allo SCT. That is, inhibitory NKR induction on alloreactive CTLs may prevent GVHD and mismatch

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Submitted November 13, 2003; accepted March 26, 2004. Prepublished online as *Blood* First Edition Paper, April 8, 2004; DOI 10.1182/blood-2003-11-3870.

Supported in part by a grant from the Idiopathic Hematological Disease and Bone Marrow Transplantation Research Committee of the Ministry of Health and Welfare of Japan; and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan; and a grant from Kirin Brewery Company (Tokyo, Japan).

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of NKRs, and ligands may be useful for induction of the GVL effect during allo SCT.[20-22]

Although granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cell (G-PBMC) grafts contain at least 10 times more T cells than do standard bone marrow grafts, the incidence and severity of acute graft-versus-host disease (aGVHD) after allogeneic peripheral blood stem cell transplantation (PBSCT) are not higher than those observed with allogeneic marrow. Also, there is a possibility that allogeneic PBSCT prevents leukemia relapse by induction of the GVL effect.[23-25] It was previously reported that G-PBMC leukapheresis products contain large numbers of $CD14^+$ cells, which suppress donor T-cell proliferation in a dose-dependent fashion.[26,27] Also, we have shown that the induction of a costimulatory molecule, CD28 responsive complex, in $CD4^+$ cells appears to be suppressed by the presence of $CD14^+$ cells in G-PBMCs.[28] Therefore, it seems useful to use G-PBMCs as a source of lymphocytes in order to manipulate cells for cell therapy to modulate GVHD and GVL. In this study, we expanded NKR-expressing T cells from donor G-PBMCs and investigated their cytolytic characteristics and regulatory functions.

Materials and methods

Donors and G-CSF mobilization

Peripheral blood stem cell donors were administered rhG-CSF (Lenograstim, 1.2 million units (MU)/10 μg, Chugai or Filgrastim, 1 MU/10 μg, Kirin-Sankyo, Japan) by subcutaneous injection at a dose of 10 μg/kg once daily for 4 to 5 days. Leukapheresis was performed from day 4 of rhG-CSF administration, and G-PBMCs were obtained from the first leukapheresis. PBMCs before administration of G-CSF (PreG-PBMC) and G-PBMC samples were cryopreserved to enable simultaneous testing.

Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies

The phycoerythrin (PE)–conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94) was obtained from Ancell (Bayport, MN), and Z199 (anti-NKG2A), ON72 (anti-NKG2D), Z231 (anti-NKp44), and C1.7 (anti-CD244) were obtained from Immunotec (Marseilles, France). Fluorescein isothiocyanate (FITC)–conjugated anti-CD3, anti-CD8 mAb, and anti–HLA-A, -B, -C mAb (G46-2.6) were purchased from Pharmingen (San Diego, CA). Anti-CD56 mAb and anti–granzyme A mAb were obtained from Becton Dickinson (BD, San Jose, CA). Anti-HLA class I mAb BRA-23/9 and W6/32 were obtained from NeoMarkers (Fremont, CA), and rat anti-HLA class I mAb (YTH862.2) was obtained from Serotec (Oxford, England). Anti-CD3 mAb OKT3 was obtained from Ortho Biotech (Raritan, NJ). Anti-NKG2C and anti-NKG2D mAb were obtained from R&D Systems (Minneapolis, MN). Intracellular granzyme A was stained using cytofix/cytoperm reagent according to the manufacturer's instructions (Becton Dickinson). The fluorescence intensity of the cells was analyzed using a FACS Calibur (Becton Dickinson). Statistical analysis was performed using Student *t* test.

Immunomagnetic cell sorting

Purified $CD14^+$ cells (> 95% $CD14^+$, as determined by flow cytometric analysis), $CD8^+$ cells (> 90% $CD8^+$), and $CD94^+$ cells (> 90% $CD94^+$) were obtained by magnetic cell sorting (MACS) using magnetic microbeads according to the manufacturer's instructions (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Induction of CD94/NKG2A on $CD8^+$ T cells by stimulation with immobilized anti-CD3 monoclonal antibody and IL-15

For coating with anti-CD3 mAb, 24-well flat-bottom plates or tissue culture flasks were preincubated with OKT3 (1 μg/mL) in 100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl buffer (pH 9.5) for 16 hours at 4°C. Seven paired PreG-PBMCs and G-PBMCs (1×10^6/mL) were cultured on 24-well plates in RPMI 1640 supplemented with 10% fetal calf serum with 5 ng/mL of recombinant human IL-15 (R&D Systems) at 37°C for 7 days. $CD8^+$ cells (500×10^3/mL) purified from G-PBMC cultures were established on 24-well plates with or without the use of 0.45-μm micropore membranes (Falcon, Becton Dickinson, Franklin Lakes, NJ). Purified $CD14^+$ cells (300×10^3) derived from the same G-PBMCs were added to the culture directly or through the membrane.

Expansion of CD94-expressing cells from G-PBMCs

Six paired PreG-PBMCs and G-PBMCs (2.5×10^6) were cultured with immobilized anti-CD3 mAb (1 μg/mL) and IL-15 (5 ng/mL) in RPMI 1640 with 5% human AB serum in T25 culture flasks for 7 days. After 5 days of culture, 5 mL of fresh medium was added. Absolute numbers of $CD94^+/CD3^+$, $CD94^+/CD8^+$, $NKG2A^+/CD3^+$, and $NKG2A^+/CD8^+$ cells on day 7 were calculated from multiplication of total number of expanded cells and the proportion of these cells in expanded cells.

PCR reaction and TCR spectratyping

First-strand cDNA synthesis was performed with 60 ng RNA, 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates (dNTPs), 2.5 μM Random 9 mer, and 0.25U/μL avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa RNA PCR Kit, Japan). Then polymerase chain reaction (PCR) amplification of the cDNA was carried out using a sense primer (5'-CAGCATGAGGGGCTACCCG-3') and an antisense primer (5'-GTGTGAGGAAGGGGGTCATG-3') for exon 4 of HLA-E.[29] A sense primer (5'-TTCGAGCAAGAGATGGCCACGGCT-3') and an antisense primer (5'-ATACTCCTGCTTGCTGATCCACAT-3') for β-actin were used as an internal standard.

For analysis of the TCR-VB repertoire, PCR amplification of the cDNA was carried out using corresponding primers to the variable regions of TCR-VB and CB.[30] Samples consisting of 1 μL of PCR product with a size standard (labeled ROX) and paraformamide were heated at 95°C for 2 minutes and then placed for a moment on ice. TCR spectratyping was performed using a capillary electrophoresis system (PRISM310 Genetic Analyzer, ABI, Foster City, CA).

Evaluation of cytolytic activity using 4-hour ^{51}Cr release assay

After 7 days of stimulation by immobilized anti-CD3 mAb with IL-15 in a T25 flask, CD94-expressing cells were purified by MACS. The cytolytic activities of purified CD94-expressing cells were tested against ^{51}Cr-labeled human malignant cell lines, patients' own leukemic cells, autologous PHA blasts, and allogeneic PHA blasts (5×10^3). K562 cells, an erythroleukemic cell line, were cultured with interferon-γ (IFN-γ) (0.2 μg/mL) for 2 days to induce HLA class I expression. An HLA-Cw3 signal peptide (VMAPRTLIL), which can bind to HLA-E, and an irrelevant B15 peptide (VTAPRTVLL)[31] were synthesized by Kurabo (Osaka, Japan) (purity, 95%). Several leukemic cell lines and solid cancer cell lines were obtained from Riken (Tsukuba, Japan).

Mixed lymphocyte culture

Responder CD94-expressing cells and CD94-depleted cells (50×10^3) were cultured with 50×10^3 irradiated (30 Gy) allogeneic, third-party PBMC stimulators in 200 μL of RPMI 1640 supplemented with 10% fetal calf serum in round-bottom 96-well plates (Corning, Corning, New York). After 2 days of incubation at 37°C in 5% CO_2, cultures were pulsed with ^3H-thymidine (1.0 μCi [0.037 MBq]/well) for the final 16 hours. The cells were then harvested, and ^3H-thymidine incorporation was measured in triplicate using a 196 gas flow counter (Packard Instrument, Downers Grove, IL). Anti–TGF-β1 mAb (25 μg/mL, R&D Systems) was added to the MLC medium using CD94-expressing cells as responders.

Measurement of cytokine concentrations

Cytokine concentrations in MLC (50×10^3 responders and 50×10^3 irradiated stimulators) after 2 days and in culture medium (50×10^3)

stimulated by phorbol myristate acetate (PMA) (10 ng/mL) and ionomycin (500 ng/mL) after 1 day were estimated. TGF-β1 was measured by using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), and other cytokines were measured by using a LiquiChip Human Cytokine System (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

NOD/SCID mice

Female 5- to 8-week-old NOD/SCID mice were purchased from Clea (Tokyo, Japan). Breeding and maintenance were performed in a micro-isolator under sterile conditions. K562 cells or CW2 colon cancer cells with or without purified CD94-expressing cells expanded from G-PBMCs were suspended in 0.5 mL phosphate-buffered saline (PBS) and injected subcutaneously into the right flanks of the NOD/SCID mice. NOD/SCID mice did not receive irradiation or anti-ASGM1 antibody.

Results

Induction of CD94/NKG2A expression on CD8$^+$ T cells in G-PBMCs by immobilized anti-CD3 monoclonal antibody stimulation with IL-15

We found that the proportion of CD94/NKG2A-expressing CD3$^+$/CD8$^+$ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation (Table 1). Although there was no difference between the proportions of CD94/NKG2A-expressing T cells in PBMCs obtained from 7 donors before administration of G-CSF (PreG-PBMCs) and after administration of G-CSF (G-PBMCs) without stimulation, the proportions of CD94/NKG2A-expressing T cells derived from G-PBMCs after 7 days of stimulation with immobilized anti-CD3 mAb both with and without IL-15 were significantly higher than the proportions of CD94/NKG2A-expressing T cells derived from PreG-PBMCs (Table 1). We also found that the proportions of CD94/NKG2A-expressing CD8$^+$ T cells that had been purified from G-PBMCs before culture were increased by immobilized anti-CD3 mAb stimulation with IL-15 (Table 2). The addition of 3×10^5 purified CD14$^+$ cells derived from the same G-PBMCs to purified CD8$^+$ T cells induced much more CD94/NKG2A expression on those purified CD8$^+$ T cells. This effect of purified CD14$^+$ cells tended to be inhibited by the use of a membrane (Table 2). These results suggest that CD14$^+$ cells play an important role in the induction of CD94/NKG2A expression on T cells and that this effect might require at least partial contact between responder cells and CD14$^+$ cells. Furthermore, it was revealed that CD94/NKG2A expression on purified CD8$^+$ T cells from G-PBMCs could be induced in our culture system. TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on CD8$^+$ T cells.[6] Several cytokines, such as IL-12 and IL-15, are known to be CD94/NKG2A-inducible cytokines.[7,32] It is possible that IL-15 induces inhibitory NKRs on CD8$^+$ T cells derived from G-PBMCs during T-cell activation by the immobilized anti-CD3 mAb.

Expansion of CD94-expressing cells from G-PBMCs

PreG-PBMCs and G-PBMCs contained almost equal numbers of CD94/NKG2A-expressing T cells before stimulation. CD94/NKG2A-expressing CD8$^+$ T cells from both PreG-PBMCs and G-PBMCs were expanded by more than 100-fold after 7 days of culture. Moreover, a significantly greater number of CD94/NKG2A-expressing T cells was obtained from G-PBMCs than from PreG-PBMCs (Table 3). CD94$^+$ cells (> 90% CD94$^+$) were obtained by MACS using magnetic microbeads, and more than 80% of CD94-expressing cells coexpressed CD8 (data not shown). The CD94-depleted cells contained only low CD94-expressing CD8$^+$ cells (mean fluorescence intensity [MFI], CD94-depleted cells vs CD94-expressing cells, 24.7 ± 7.2 vs 234.1 ± 30.5, n = 7). In contrast, most of these CD94$^+$ cells did not express CD56. Furthermore, these CD94$^+$ cells contained granzyme A, which is an important enzyme for induction of apoptosis of target cells in the cytoplasm.[33] Also, CD94-expressing cells expanded from G-PBMCs had a large repertoire of TCR-Vβ, as revealed by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis using 26 kinds of TCR-Vβ primer pairs (data not shown). These expanded CD8$^+$ T cells expressed NKG2D and CD244 but did not express CD158a, CD158b, NKB1, CD161, nor NKp44 (data not shown).

Characteristics of cytolytic activities of CD94-expressing cells expanded from G-PBMCs against K562 leukemic cells

We investigated the characteristics of cytolytic activity of CD94-expressing cells expanded from donor G-PBMCs. The cytolytic activity level of purified CD94-expressing cells detected by a standard 4-hour ^{51}Cr release assay against HLA class I–deficient K562 cells was found to be always higher than that of CD94-depleted cells and also higher than that against autologous PHA blasts (Figure 1A). Furthermore, the cytolytic activity level of CD94-expressing cells against allogeneic PHA blasts was as low as that against auto PHA blasts (Figure 1B). HLA class I expression is inducible on K562 cells by IFN-γ. The MFIs of HLA class I molecules on untreated K562 and IFN-γ–treated K562 cells were 21.8 ± 14.6 (n = 6) and 90.1 ± 25.5 (n = 6), respectively. Although we did not show the surface expression level of HLA-E on HLA class I–expressing cells, we could show HLA-E mRNA induction in HLA class I–expressing K562 cells in an RT-PCR experiment (data not shown). Therefore, the leader peptide of HLA class I stabilizes HLA-E expression and subsequently may be able to induce a higher level of HLA-E expression on HLA class I–expressing cells. The cytolytic activity of CD94-expressing cells against IFN-γ–treated K562 cells was attenuated compared with

Table 1. Proportion of CD94/NKG2A-expressing cells in paired PreG- and G-PBMC before and after stimulation by anti-CD3 monoclonal antibody

	Before		After anti-CD3 stimulation		Anti-CD3 and IL-15	
Surface marker	PreG	G-PBMC	PreG	G-PBMC	PreG	G-PBMC
CD94$^+$/CD3$^+$	5.7 ± 3.1	4.6 ± 1.9	8.4 ± 4.4	17.2 ± 8.7*	24.2 ± 10.4	32.8 ± 8.2†
CD94$^+$/CD8$^+$	2.6 ± 1.6	2.7 ± 1.2	7.3 ± 4.0	15.2 ± 8.4*	17.6 ± 10.0	24.2 ± 5.6‡
NKG2A$^+$/CD3$^+$	1.8 ± 1.4	1.8 ± 0.7	3.0 ± 1.9	6.3 ± 5.0	11.3 ± 7.1	16.9 ± 7.7‡
NKG2A$^+$/CD8$^+$	0.5 ± 0.3	0.4 ± 0.5	2.3 ± 1.3	5.1 ± 4.3	8.6 ± 3.1	13.6 ± 6.3*

Values indicate the percentage of CD94 or NKG2A-expressing cells (means ± SDs, n = 7). Significant difference were noted when comparing the value of PreG and G-PBMC after stimulation with and without IL-15 (*$P < .01$; ‡$P < .05$, †$P < .1$).

Table 2. Induction of CD94/NKG2A expression on purified CD8+ cells from G-PBMC

Surface marker	Before	After	Addition of CD14+ cells: Without membrane	Addition of CD14+ cells: With membrane
CD94+/CD8+	1.7 ± 0.8	9.1 ± 5.8*	19.7 ± 9.3†	11.7 ± 6.0‡
NKG2A+/CD8+	0.4 ± 0.3	3.0 ± 2.7†	10.1 ± 6.8†	5.0 ± 3.5‡

Values indicate the percentage of CD94 or NKG2A-expressing cells after anti-CD3 stimulation in the presence of IL-15 (means ± SDs, $n = 7$). Significant differences were noted when comparing the value of before and after stimulation; CD8+ cell only and addition of purified 3×10^5 CD14+ cells; without membrane; and the contact inhibition by the membrane.

*$P < .01$.

†$P < .05$.

‡$P < .1$.

that against untreated K562 cells. Furthermore, HLA-Cw3 peptide (0.3 mM), which is a signal sequence of HLA-C and makes a complex with HLA-E as a ligand for CD94/NKG2A, suppressed the cytolytic activity of CD94-expressing cells against HLA class I–expressing K562 cells. The suppressive effect of Cw3 peptide was higher than that of an irrelevant B15 peptide. In contrast, anti-NKG2A mAb (10 μg/mL) restored the HLA class I protective effect against IFN-γ–treated K562 cells (Figure 2A). Also, anti–HLA class I mAbs (G46-2.6 and W6/32, 20 μg/mL) restored the cytolytic activity of CD94-expressing cells against IFN-γ–treated K562 cells that had increased mRNA of HLA-E, while other anti–HLA class I mAbs (YTH862.2 and BRA-23/9, 20 μg/mL) did not have any effect (Figure 2B). Furthermore, anti-NKG2D mAb suppressed the cytolytic activity of CD94-expressing cells against K562 cells, while anti-NKG2C mAb and anti-CD244 mAb did not have any effect (20 μg/mL) (Figure 2C).

Cytolytic activities of CD94-expressing cells against various leukemic cell lines, solid cancer cell lines, and the patient's leukemic cells

We analyzed the cytolytic activities of CD94-expressing cells against 7 human leukemic cell lines and 3 solid cancer cell lines. Cytolytic activities against HLA class I^{low} cells (MFI < 50; K562 and CW2 colon cancer cells) were more than 30% (effector-to-target, 10:1). On the other hand, cytolytic activities against HLA class $I^{intermediate}$ cells (50 < MFI < 150; HL60, KCL22, HEL, and U937 leukemic cells) were 20% to 30%, and cytolytic activities against HLA class I^{high} cells (MFI > 200; J111 and BALL-1 leukemic cells and RC2 and 3TKB renal cancer cells) were less than 10% (data not shown).

We then investigated the cytolytic activities of HLA-matched donor CD94-expressing cells against the patient's chronic myelogenous leukemia (CML) cells derived from bone marrow cells before allo SCT in the chronic phase and against the patient's leukemic cells of CML myeloid blastic crisis (CML-BC), acute myelogenous leukemia (AML, M2), acute lymphocytic leukemia (ALL, L2), Ph1-positive ALL, adult T-cell leukemia (ATL), another AML (M2), and myelodysplastic syndrome (MDS) overt leukemia. Expanded and purified CD94-expressing cells derived from each donor attacked K562 cells and the patients' own leukemic cells to varying degrees, depending on the type of tumor (the tumors having different expression levels of HLA class I and probably having different expression levels of adhesion molecules and stimulatory NKR ligands) but did not attack auto PHA blasts (Figure 3A). Also, allogeneic third-party CD94-expressing cells attacked the patients' primary AML (M0, M2, M4) and CML (CP and BC) leukemic cells. However, these cells did not attack ALL (L1) cells, ATL cells, lymphoblastic leukemia lymphoma (LBL) cells, auto PHA blasts, or allo PHA blasts. Anti–HLA class I mAb partially restored this killing activity against ALL (Ph1) leukemic cells, auto PHA blasts, and allo PHA blasts (Figure 3B-C). These expanded donor and allogeneic CD94-expressing cells attacked HLA class I molecule–lacking K562 leukemic cells and also the patient's leukemic cells that were not HLA class I^{high} cells (MFI < 200) as PHA blasts.

Proliferation in MLC and cytokine productive capacity of CD94-expressing cells

Proliferation of T cells detected by ^{3}H-thymidine incorporation was suppressed in MLC using CD94-expressing cells as responders compared with that in the case of using CD94-depleted cells as responders (2697 ± 1124 vs 6586 ± 2283 cpm, $P < .01$, $n = 6$). TGF-β1 concentration in MLC medium using CD94-expressing cells as responders was significantly higher than that in MLC medium using CD94-depleted cells as responders (383.2 ± 144.3 vs 236.4 ± 89.6 pg/mL, $P < .05$, $n = 8$). IL-2 and IFN-γ concentrations in MLC medium were not significantly different. On the other hand, IL-2 concentration in culture medium stimulated by PMA and ionomycin using CD94-depleted cells was significantly higher than that in culture medium using CD94-expressing cells (13 485.9 ± 10 640.4 vs 1767.7 ± 223.8 pg/mL, $P < .05$, $n = 8$). Furthermore, it was revealed that anti–TGF-β1 mAb could restore T-cell proliferation in MLCs using CD94-expressing cells as responders (2697 ± 1124 vs 3470 ± 1262 cpm, $P < .01$, $n = 6$).

Inhibition of growth of K562 leukemic cells and CW2 colon cancer cells by CD94-expressing cells in NOD/SCID mice

NOD/SCID mice were coinjected subcutaneously with K562 cells and purified CD94-expressing cells expanded from G-PBMCs. CD94-expressing cells inhibited the growth of K562 cells completely (ratio of CD94-expressing cells to K562 cells: 1×10^7 to 2×10^7) in NOD/SCID mice (Figure 4A). CD94-expressing cells also inhibited the growth of CW2 colon cancer cells completely (ratio of CD94-expressing cells to CW2 cells: 0.5×10^7 to 1×10^7) in NOD/SCID mice (Figure 4B).

Table 3. Expansion of CD94/NKG2A-expressing cells from paired PreG- and G-PBMC

Surface marker	Before, mean absolute no. cells ± ×10⁶: PreG	Before, mean absolute no. cells ± ×10⁶: G-PBMC	After stimulation, mean absolute no. cells ± ×10⁶ (mean fold expression): PreG	After stimulation, mean absolute no. cells ± ×10⁶ (mean fold expression): G-PBMC
CD94+/CD3+	0.048 ± 0.026	0.041 ± 0.012	2.87 ± 1.38 (59.8)	5.51 ± 2.62* (134.4)
CD94+/CD8+	0.020 ± 0.007	0.010 ± 0.004	2.63 ± 1.25 (131.5)	5.20 ± 2.36† (520.0)
NKG2A+/CD3+	0.021 ± 0.012	0.018 ± 0.010	2.09 ± 1.10 (99.5)	4.20 ± 2.44* (233.3)
NKG2A+/CD8+	0.0052 ± 0.0036	0.0036 ± 0.0012	1.90 ± 1.02 (365.4)	3.98 ± 2.21* (1105.6)

Cultures were started from 2.5×10^6 mononuclear cells. Values indicate absolute number of cells before and after culture. Significant differences were noted when comparing the value of PreG and G-PBMC after stimulation (*$P < .05$, †$P < .01$).

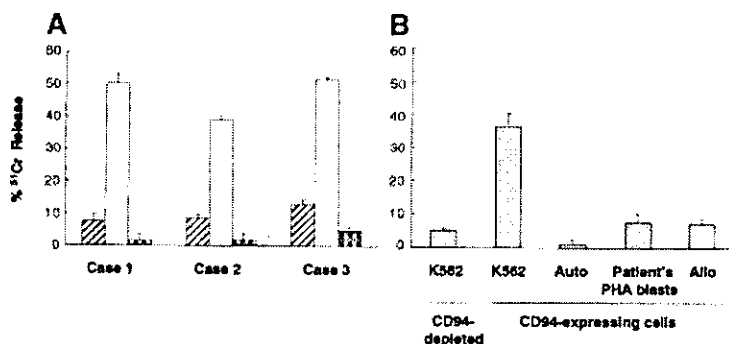

Figure 1. Cytolytic activities of CD94-expressing cells against K562 leukemic cells and PHA blasts. (A) Cytolytic activities of CD94-depleted cells (▨) and CD94-expressing cells expanded from G-PBMCs against K562 cells (□) and autologous PHA blasts (□). (B) Cytolytic activities of CD94-depleted cells and CD94-expressing cells expanded from G-PBMCs against K562 cells, autologous PHA blasts, patient's PHA blasts, and allogeneic third-party PHA blasts. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

Discussion

In this study, we found that the proportion of CD94/NKG2A-expressing CD3$^+$/CD8$^+$ T cells in G-PBMCs was increased after immobilized anti-CD3 mAb stimulation with IL-15. We also found that CD14$^+$ cells derived from G-PBMCs play an important role in the induction of CD94/NKG2A expression on purified CD8$^+$ T cells. Therefore, CD8$^+$ T cells derived from G-PBMCs could express CD94/NKG2A after stimulation. Also, we were able to expand CD94-expressing CD8$^+$ T cells from donor G-PBMCs by more than 500-fold. The absolute number of CD94-expressing T cells after stimulation from G-PBMCs was significantly higher than that from PreG-PBMCs. It is possible that a greater number of CD14$^+$ cells in G-PBMCs than in PreG-PBMCs can stimulate the first signal through TCR. This TCR engagement has been reported to play an important role in the induction of inhibitory NKRs on CD8$^+$ T cells.[6] Although we showed a CD94-inducing effect of CD14$^+$ cells in a contact-dependent manner, other factors such as cytokines may be implicated in this effect. Also, it is not clear enough whether G-CSF has an effect on progenitor cells of CD94/NKG2A-expressing cells. Nevertheless, G-PBMCs, which are easy to obtain and store at the time of PBSC collection from the donor, may be a useful source for the expansion of inhibitory NKR-expressing cells.

These expanded and purified CD94-expressing cells had CD8 expression but not CD56 expression on their surfaces. Also, these CD94-expressing cells contained granzyme A in the cytoplasm and had a large repertoire of TCR-Vβ as revealed by RT-PCR analysis using 26 kinds of TCR-Vβ primer pairs. Furthermore, these expanded CD8 T cells did not express other killer cell immunoglobulin-like receptors (KIRs) such as CD158a, CD158b, or NKB1 or NK cell–activating markers such as CD161 or NKp44, but they did express NK cell–activating receptors NKG2D and CD244. Therefore, these cells have both inhibitory receptors (CD94/NKG2A) and activating receptors (NKG2D). The cytolytic activity of CD94-expressing cells depends at least partially on NKG2D-activating NKR, because anti-NKG2D mAb suppressed this activity. However, it is possible that other receptors that were not analyzed in this study are involved in the killing activity.

HLA-E, a CD94/NKG2A ligand, preferably bound to a peptide derived from the signal sequences of most HLA-A, -B, -C, and -G and was also up-regulated by these peptides.[31] We investigated the characteristics of cytolytic activities of CD94-expressing cells using IFN-γ–induced HLA class I molecule–expressing K562 cells that had increased mRNA of HLA-E. HLA-C signal peptide was found to suppress the cytolytic activity of CD94-expressing cells against IFN-γ–induced HLA class I molecule–expressing K562 cells. Also, anti-NKG2A mAb and some anti–HLA class I mAbs partially restored the cytolytic activity of CD94-expressing cells against HLA class I molecule–protected K562 cells. In addition, results of analysis of the cytolytic activities of CD94-expressing cells against 10 malignant cell lines, including 3 solid cancer cell lines, indicated that this killing activity roughly depended on the expression of HLA class I molecules on the cell surface. However, the cytolytic activity of CD94-expressing cells does not depend entirely on the expression of HLA class I molecules. The cytolytic activity of CD94-expressing cells may be regulated by the balance among the expression levels of HLA class I, HLA-E itself, and certain molecules on target cells.

We also investigated the cytolytic activities of CD94-expressing cells against 17 patients' primary leukemic cells. Donor and allogeneic CD94-expressing cells could attack patients' CML cells and AML cells but could not attack some patients' leukemic cells such as ATL cells, which had high expression levels of HLA class I molecules. Also, the addition of anti–HLA class I mAb induced

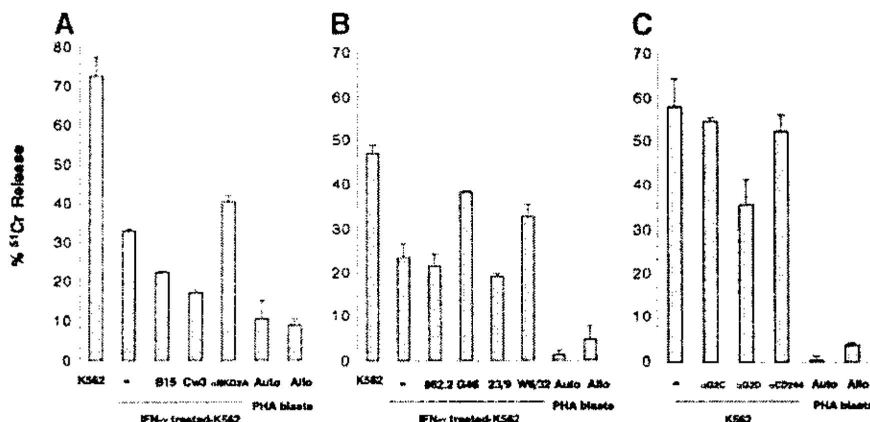

Figure 2. Characteristics of cytolytic activities of CD94-expressing cells against K562 leukemic cells. (A) Cytolytic activities of CD94-expressing cells against untreated K562 cells, IFN-γ–treated K562 cells, IFN-γ–treated K562 cells with HLA-B15 peptide (0.3 mM), with HLA-Cw3 peptide (0.3 mM), and with anti-NKG2A mAb (10 μg/mL) against autologous PHA blasts and allogeneic PHA blasts. (B) Cytolytic activities of CD94-expressing cells against untreated K562 cells, IFN-γ–treated K562 cells, and IFN-γ–treated K562 cells with anti–HLA class I mAbs (YTH862.2, G46-2.6, BRA-23/9, and W6/32, 20 μg/mL) and against autologous PHA blasts and allogeneic PHA blasts. (C) The cytolytic activities of CD94-expressing cells against K562 cells, against K562 cells with anti-NKG2C, anti-NKG2D, and anti-CD244 mAbs (20 μg/mL), against autologous PHA blasts and allogeneic PHA blasts. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

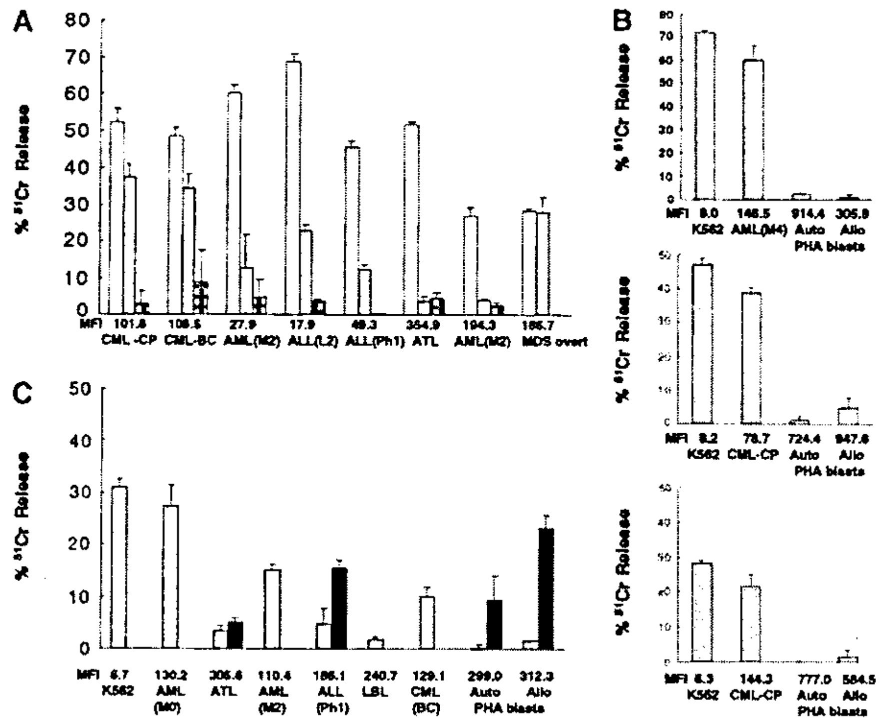

Figure 3. Cytolytic activities of CD94-expressing cells expanded from G-PBMCs against the patients' leukemic cells. (A) Cytolytic activities of HLA-matched donor CD94-expressing cells expanded from 8 different donor G-PBMCs against K562 cells (□), the patients' own leukemic cells (□), and autologous PHA blasts (□). (B, C) Cytolytic activities of allogeneic third-party CD94-expressing cells expanded from 4 different donor G-PBMCs against K562 cells, the patients' primary leukemic cells, autologous PHA blasts, and allogeneic PHA blasts. Addition of anti–HLA class I mAbs (W6/32, 20 μg/mL) (■). MFIs of HLA class I on target cells are indicated. The data represented are the means ± SDs (effector-to-target ratio is 10:1).

restoration of the cytolytic activity of CD94-expressing cells against PHA blasts and ALL (Ph1) but not ATL cells. Although these CD94-expressing cells attacked HLA class $I^{low\text{-}intermediate}$ patients' leukemic cells, the killing activity varied, depending on the type of leukemia. Patients' leukemic cells have different expression levels of HLA class I, and they may have different expression levels of other regulatory molecules for the killing activities of CD94-expressing cells. Therefore, not only HLA class I molecules on leukemic cells but also other molecules such as adhesion molecules and stimulatory NKR ligands such as MHC class I chain–related protein (MIC[34]) and activating molecules on effector cells might be important for the regulation of these killing activities.

In vivo analysis revealed that CD94-expressing cells prevented the growth of K562 leukemic cells and also CW2 colon cancer cells in NOD/SCID mice. These models suggest that CD94-expressing cells may therefore have a graft-versus-leukemia/tumor effect.

In addition, the CD94-expressing cells exhibited low proliferative capacity in MLC and high TGF-β1 productivity with attenuated IL-2 productivity. These cells therefore have low responsiveness to alloantigens and may also have a suppressive effect on HLA class I–induced alloresponse.

We previously reported increased expression of CD158 and CD94/NKG2A on T cells in chronic GVHD patients with good prognosis and showed that these inhibitory NKR-expressing cells have a suppressive effect on allogeneic response in MLC.[16-19] Therefore, inhibitory NKR expression during allogeneic stimulation after allo SCT may play an important role in modulation of GVHD. Based on clinical and experimental data, we speculate that these inhibitory NKR-expressing cells have a GVL/T effect against leukemic cells and tumor cells that have decreased expression levels of HLA class I molecules and do not enhance GVHD against host cells that have normal expression levels of HLA class I molecules.

Elucidation of cytolytic characteristics, proliferative characteristics, and cytokine productivity of inhibitory NKR-expressing cells might provide clues about how to control the delicate balance between GVHD and GVL. It may be possible to use expanded CD94-expressing cells from donor G-PBMCs, which contain a large number of T cells, for allogeneic cell therapy instead of naive donor lymphocyte infusion to induce the GVL effect without enhancing GVHD. Donor G-PBMCs, which are an alternative stem cell source for allogeneic stem cell transplantation, might also be a useful source of lymphocytes for expanding NKR-expressing cells for cell therapy for some patients whose leukemic cells and tumor cells have escaped from allogeneic recognition by usual cytotoxic T cells because of the low expression level of HLA class I molecules.

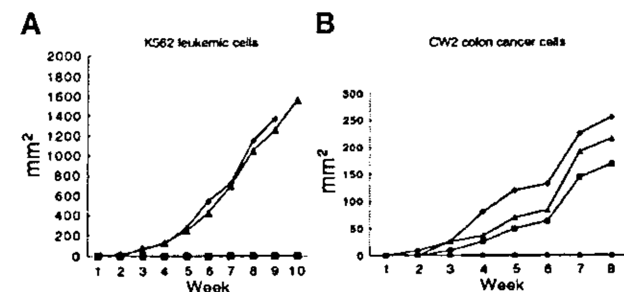

Figure 4. CD94-expressing cells expanded from G-PBMCs prevent growth of K562 leukemic cells and CW2 colon cancer cells in NOD/SCID mice. (A) Mice were subcutaneously injected with 2 × 10^7 K562 cells only (♦ and ▲; died after 9 and 13 weeks, respectively) or with 1 × 10^7 CD94-expressing cells (2 mice, ■, survived more than 30 weeks). (B) Mice were subcutaneously injected with 1 × 10^7 CW2 colon cancer cells only (♦, ▲, and ■; died after 9, 9, and 14 weeks, respectively) or with 0.5 × 10^7 CD94-expressing cells (3 mice, ●, survived more than 30 weeks).

Acknowledgments

We thank Ms M. Yamane, Ms M. Mayanagi, and Ms R. Miura for their technical assistance.

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Hepatology Research 30 (2004) 175–181

Hepatology Research

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Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein

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Received 24 May 2004; received in revised form 11 August 2004; accepted 6 September 2004
Available online 13 November 2004

Abstract

Hepatocyte growth factor (HGF) stimulates liver regeneration and has the potential to be a therapeutic agent for fatal liver diseases, including fulminant hepatic failure and liver cirrhosis. In this study, we investigated the pharmacokinetics of recombinant human HGF, which will be soon available for clinical applications. When recombinant human HGF (0.1 mg/kg) was administered intravenously to normal rats, serum levels of human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the bolus injection, followed by a decrease with a half-life of 2.4 min. Recombinant HGF administered intravenously was distributed primarily to the liver and induced c-Met tyrosine phosphorylation in liver tissues. In comparison, rats given recombinant human HGF via the portal vein exhibited lower serum HGF and an increase in hepatic distribution. Additionally, when compared with normal rats, those with 70% partial hepatectomy or liver cirrhosis showed an increase in serum levels of human HGF with a prolonged half-life. These results suggest that, despite a short half-life, bolus injection of recombinant human HGF may induce therapeutic effect in patients with fatal live disease, and that the dose of this recombinant protein should be modulated according to the degree of liver injury.
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Keywords: Hepatocyte growth factor; Pharmacokinetics; Tissue distribution; Serum HGF

1. Introduction

Hepatocyte growth factor (HGF) was originally purified from the plasma of patients with fulminant hepatic failure [1,2], and is one of the major agents that promote hepatocyte proliferation. HGF also functions as a pleiotropic factor, acting as a mitogen, motogen, and morphogen for a variety of epithelial cells (e.g. kidney, intestinal and bronchial cells) as well as endothelial cells by ligation of the c-Met receptor at the cell membrane [3–8]. Additionally, recent investigations have demonstrated that HGF inhibits apoptosis [9–15], and that fibrotic changes in various organs, including the liver and kidneys, were ameliorated by treatment with HGF [16–21]. Thus, HGF plays an important role in the regeneration and repair of injured tissues.

We have previously established an enzyme-linked immunosorbent assay (ELISA) to measure human HGF in serum, and reported that levels of serum human HGF increased in patients with various liver diseases [22]. We have also demonstrated that the measurement of serum HGF is useful for outcome prediction in fulminant hepatic failure [23]. Recombinant human HGF will soon be available for patients with fatal liver diseases, including fulminant hepatic failure, small-for-size grafts in living donor liver transplantation (LDLT), and liver cirrhosis. Since HGF functions as an anti-

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doi:10.1016/j.hepres.2004.09.002

apoptotic or anti-fibrotic factor as well as an agent stimulating hepatocyte proliferation, administration of recombinant HGF is considered to not only induce liver regeneration, but also inhibit disease progression and ameliorate liver cirrhosis. In the present study, we investigated the pharmacokinetics of recombinant human HGF administered intravenously or via the portal vein. We show here that intravenous injection of recombinant human HGF in a bolus induced an increase in the serum levels of human HGF, and that intravenously administered HGF was primarily distributed to the liver. Conversely, compared with intravenous administration, portal vein injection of recombinant human HGF increased hepatic distribution of the protein, whereas serum HGF levels were reduced. We also demonstrate that, despite the short half-life, the single intravenous injection of recombinant HGF induced tyrosine phosphorylation of c-Met in liver tissues.

2. Materials and methods

2.1. Animals

Eight-week-old male Wistar rats were obtained from Japan SLC Inc. (Shizuoka, Japan). The animals were maintained under constant room temperature (25 °C) and given free access to water and the indicated diet throughout the study. The protocol for animal studies was approved by the ethical committee of the Graduate School of Medicine, Kyoto University (Kyoto, Japan). All animal experiments were performed after a 1-week acclimation period on a standard diet.

Seventy percent partial hepatectomy was performed according to a modification of the method of Higgins and Anderson [24]. The rats were anesthetized with ether and a two-thirds partial hepatectomy was performed. To induce liver fibrosis, the rats were fed a choline-deficient, L-amino acid-defined (CDAA) diet (Dyets Inc., Bethlehem, PA) for 30 weeks. The development of cirrhosis was confirmed by macroscopic inspection and histological examination [25].

2.2. Measurement of serum human HGF

A silicone-rubber catheter (0.5 mm × 1.0 mm o.d.) was inserted into the jugular vein and saline was administered continuously via the catheter using an infusion pump (0.1 ml/h) to prevent obstruction. Recombinant human HGF (0.1 mg/kg) was injected into inguinal vein or splenic vein in less than 10 s, and sequential blood samples were obtained via the catheter 5, 10, 20, 30, 60, 90 and 120 min after the injection.

2.3. Preparation of tissue extracts

Tissue extracts were prepared as previously described [26] with a slight modification. The various tissues were excised 5 min after the intravenous or intraportal injection of recombinant human HGF (0.1, 0.03 or 0.01 mg/kg), and the wet weight of tissue samples was determined. Fresh tissues were homogenized in cold Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.4% EDTA-2Na and 500 units/ml of aprotinin. The homogenates were centrifuged at 9000 × g for 20 min at 4 °C, and then at 105,000 × g for 1 h at 4 °C. The supernatants were used for measurement of HGF levels and Western blot analysis.

2.4. Measurement of HGF in sera and tissue extracts

HGF levels in serum and tissue extracts were determined by a commercially available ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan), in which only human HGF, but not rat HGF, is detected [22,26].

2.5. Western blotting

Tyrosine phosphorylation of c-Met was evaluated by Western blotting. Liver tissues were solubilized in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A. Post-nuclear supernatants were precleared with protein A-agarose and immunoprecipitated with anti-c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-agarose. Immunoprecipitated materials were washed five times with 0.1% NP40 and 0.05% sodium deoxycholate and eluted by boiling in Laemmli sample buffer (Bio-Rad, Hercules, CA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. After blocking membranes with 1% bovine serum albumin, filters were incubated with horseradish peroxidase-conjugated anti-phosphotyrosine antibody and subjected to ECL Western blotting detection (Amersham Life Sciences, Buckinghamshire, England).

3. Results

3.1. Changes in serum levels of recombinant human HGF in normal, hepatectomized, and cirrhosis rats

We examined changes in serum levels of human HGF following bolus injection of recombinant human HGF using ELISA (Fig. 1). Our ELISA could not detected endogenous rat HGF. When recombinant human HGF (0.1 mg/kg) was injected into normal rats via inguinal veins in less than 10 s, the level of serum human HGF increased to 89.7 ± 20.6 ng/ml 5 min after the injection (Fig. 1A). Recombinant human HGF disappeared from serum with a half-life of 2.4 min, and the serum HGF decreased to 0.65 ± 0.13 ng/ml after 120 min. These findings indicate that the bolus injection of recombinant HGF induced a considerable increase in serum human HGF, followed by disappearance with a short half-life.

Recombinant human HGF will soon be administered to patients with fatal liver disease, including small-for-size grafts