response with a median follow-up of 6.5 years. Despite the intensive therapy, Grade 3–4 neutropenia was observed only in two patients. This may reflect that fact that the patients in this cohort were relatively young (a median age of 27 years). Such intensive therapy may be best reserved for salvage in adult patients with resistant or progressive MS LCH [16].

In the present pilot study, 10 of the 14 patients responded and five patients maintained a continuous response with a median follow-up of 2.8 years. A response was obtained in all of the patients with SS disease and in half of the patients with MS disease. The Special C regimen was originally prepared to give it safely at the outpatient clinic. Therefore, it was a surprise that we observed a fairly high % of ≥grade 3 adverse events in our cohort, even including a fatal case. This may mean that, especially in the treatment of adult LCH, we have to be careful for various co-morbidities which could affect the patients' outcome associated with therapeutic procedures for LCH. In SS patients, the response rate is comparable to those achieved with the 2CdA and MACOP-B regimens, but the response rate of the MS patients may be slightly more inferior (Table 3). However, the Special C regimen is particularly significant because it is an ambulatory treatment. It may be necessary to intensify the treatment for patients with MS disease, but careful consideration should be given when the patient is elderly because two of the three patients over 60 years of age died from infection during subsequent treatments for refractoriness and reactivation.

A multicenter phase II study with a large number of patients with multifocal LCH that allows risk stratification is needed to establish a more efficient and less toxic regimen. To promote this, closer cooperation between pediatric and adult hematologists and experts in other fields (such as dermatology, orthopedics and neurology) is essential. Such a study may improve the quality of life of adult patients with LCH.

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Conflict of interest The authors declare that they have no conflicts of interest.

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LETTER TO THE EDITOR

Feasibility of autologous bone marrow mesenchymal stem cells cultured with autologous serum for treatment of haemophilic arthropathy

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Patients with severe haemophilia frequently experience spontaneous intra-articular haemorrhages, mainly in the ankles, knees and elbows. Over the long term, repeated episodes of haemarthrosis may cause irreversible damage to the joint, leading to haemophilic arthropathy, a polyarticular disease characterized by joint stiffness, chronic pain and a severely limited range of motion [1]. The progression from recurrent haemarthrosis to arthropathy is caused by inflammatory synovitis and cartilage destruction. Haemosiderin deposition into synovial tissues induces proliferation of the synovium and neovascularization of the subsynovial layer, which results in an inflamed, villous, synovial tissue. This friable and highly vascular synovium is more susceptible to further haemorrhage with minimal stress, which sets up a vicious cycle that is difficult to break [2]. Then, the articular cartilage defects occur and finally go into the cartilage destruction.

If conservative management (analgesics, orthotics and physical therapy) for the haemophilia patients who develop chronic synovutis and arthropathy fails, surgical interventions should be considered at a relatively young age. The most common surgical procedures are synovectomy, arthrodesis and total joint arthroplasty. So far, however, the regenerative medicine aiming at the repair of articular cartilage defects has not been done in the patients with haemophilic arthropathy.

We previously performed the transplantation of mesenchymal stem cells (MSCs) for the patients with osteoarthritis (OA) to repair their articular cartilage defects. MSCs generated from autologous BM blood (BMMSCs) were transplanted to the area of the articular cartilage defects with type I collagen gel. After the transplantation, the clinical outcomes were significantly improved [3–7]. Another groups also reported the repair of the articular cartilage defects with autologous BMMSC transplantation [8]. In addition, we reported that no abnormal tumours appeared in the 45 operated joints of 41 patients who received the transplantation of autologous BMMSCs between 1998 and 2008 by our groups (follow-up duration: mean, 75 months; range 5–137 months), demonstrating that autologous BMMSC transplantation is a safe procedure [4].

Therefore, in patients with haemophilic arthropathy, repair of the articular cartilage defects with autologous BMMSCs is also expected to result in great advantage, which includes the relief of pain and swelling, and increase of joint motion, and we planed the clinical trial for the treatment of haemophilic arthropathy by the autologous BMMSC transplantation. However, there have been no reports on the potentials of BMMSCs in haemophilia patients to proliferate *in vitro* and to differentiate into chondrocytes. Then, before the clinical trial, we needed to confirm the capabilities of BM cells in haemophilia patients to produce MSCs and the potential of the BMMSCs to differentiate into chondrocytes.

For this purpose, this study was done with the approval by Ethics Committee of The Institute of Medical Science, The University of Tokyo (#19–10). We harvested 4 mL of BM samples from iliac bones of three haemophilia patients and one healthy volunteer after obtaining written informed consents. BM samples from three patients with haemophilia A, who had experienced orthopaedic surgery, were harvested during the general anaesthesia. BM sample from a healthy adult was obtained under local anaesthesia. Autologous serum from each donor of BM samples was collected in other day prior to the BM harvest.

Before 2001, we added foetal bovine serum (FBS) into the medium for BMMSC culture [3,4,9], but the issue of bovine spongiform encephalopathy changed our strategy not to use FBS in the culture of human BMMSCs for the therapeutic transplantation. Since then, considering this issue and the problem of transmitted infectious pathogen from allogeneic products, we used autologous serum instead of FBS or allogeneic human serum for the clinical application of human BMMSCs. Indeed, we experienced no cases who suffered with the infection in 41 patients who received the transplantation of autologous BMMSCs in our groups [4].

In the present culture, as described previously, 4 mL of harvested BM blood was divided into halves, and each 2 mL of BM blood was cultured in 75-cm^2 flask with culture media, which consist of Dulbecco's modified Eagle's medium and 15% volume of autologous serum [4,5]. After 3 days of culture, when attachment of cells was observed, the medium was exchanged, and red blood cells and non-adherent cells were discarded with the medium. After 10–12 days, adherent cells achieved subconfluence, and were passaged to expand in culture. When the adherent cells achieved confluence after another 10–12 days, we collected the cultured cells, which are supposed to be used in the clinical trial, and were processed for the experiments.

Morphology of the cultured cells from BM cells of three haemophilia patients was spindle-shaped like MSCs, and almost same as those from a healthy adult (Fig. 1a). Flow cytometric analysis revealed that cultured cells were positive for CD29, CD44, CD13, CD73, CD90 and CD105, but negative for CD45 and CD14 (Fig. 2). These results were identical with BMMSCs reported previously [10]. When induced to differentiate into chondrocytes using

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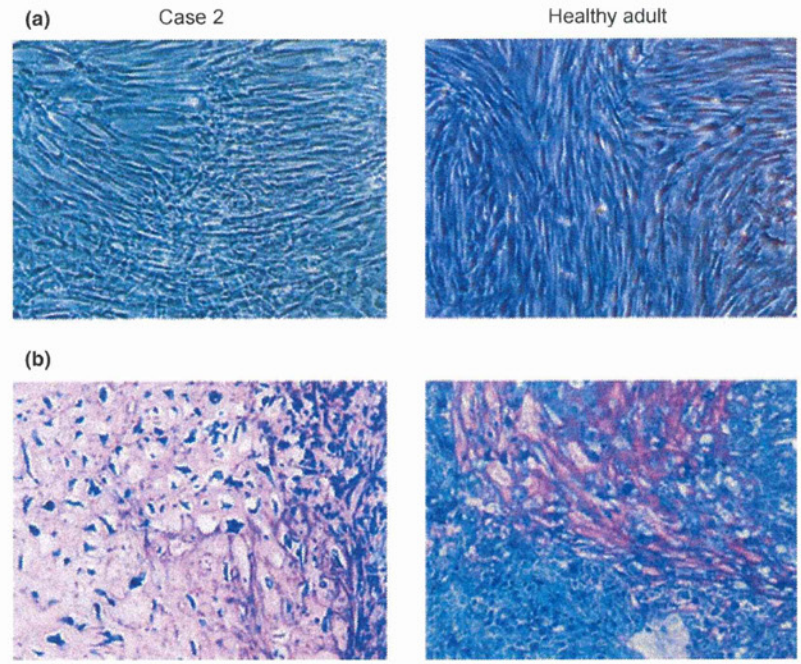

Fig. 1. Mesenchymal stem cells of BM from a haemophilia patient (case 2) and a healthy adult. (a) Morphology of the cultured cells from a haemophilia patient (case 2) and a healthy adult. They were spindle-shape like MSCs (×100). (b) Chondrocyte differentiation of BMMSCs from a haemophilia patient (case 2) and a healthy adult. Cells were stained with toluidine blue, and the stained cells were chondrocytes.

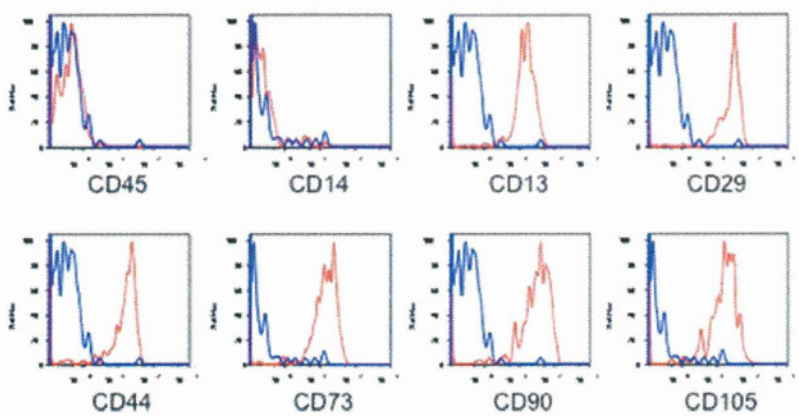

Fig. 2. Flow cytometric profiles of BMMSCs derived from a haemophilia patient (case 3). Cultured cells were stained with phycoerythrin (PE)-conjugated CD45, CD13, CD29, CD44 and CD90, and fluorescein isothiocyanate (FITC)-conjugated CD14, CD73 and CD105. Red and blue lines indicate data from the sample of case 3 and negative control respectively (×200).

NH ChondroDiff Medium (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions, the cultured cells from BM cell of haemophilia patients and healthy adult generated toluidine blue-positive chondrocytes (Fig. 1b). This result indicated that MSCs capable of differentiating into chondrocytes were generated from BM blood in haemophilia patients, similarly to those in a healthy adult.

All the cell numbers of MSCs derived from 2 mL of BM blood in three haemophilia patients were more than one million as well as a healthy adult (Table 1). Our previous experience demonstrated that 10 million of BMMSCs were enough to repair substantial range of articular cartilage defect in OA patients [3]. Consequently, we need to obtain more than 20 mL of BM blood from each patient to repair articular cartilage defects in haemophilic arthropathy, and it is possible under local anaesthesia.

In addition, chromosomal analysis revealed that the cultured cells from BM cells of three haemophilia patients had normal karyotype (Table 1), suggesting little possibility of the transformation of BMMSCs during the present culture. Thus, MSCs capable of proliferating *in vitro* and differentiating into chondrocytes were safely generated from BM cells of haemophilia patients similarly with those from healthy adult, indicating the feasibility of the regenerative medicine using BMMSCs to repair articular cartilage defects in the patients with haemophilic arthropathy.

Table 1. Characteristics of patients and a healthy adult, and their BMMSCs.

Case	1	2	3	Healthy adult
Age	23	20	19	55
Type of haemophilia	A	A	A	–
Cell number/2 mL BM blood*	1.5×10^6	1×10^6	1×10^6	1×10^6
Karyotype of MSC†	46, XY (10/10)	46, XY (10/10)	46, XY (3/3)	46, XY (5/5)
Chondrocyte formation	+	+	+	+

*The number indicates the average of two cultures of 2 mL of BM blood.
†The number in each parenthesis indicates the number of cells analyzed.

Some of adult patients with haemophilia have chronic viral infection. To apply the regenerative medicine using BMMSCs to such patients, it is important to validate that our culture system of BMMSCs does not provoke the reactivation of the viruses. This possibility is now under investigation.

Besides the transplantation of autologous BMMSCs, autologous chondrocyte implantation (ACI) may be considerable for the repair of articular cartilage defects in the patients with haemophilic arthropathy. However, ACI needs the excision of healthy cartilage tissue even though it is in nonweight bearing areas. The excision procedure is too invasive to operate upon haemophilia patients because they have a significant bleeding tendency. In this regard, as autologous BMMSC transplantation is less invasive, it is more feasible for the treatment of haemophilic arthropathy than AIC.

As mentioned above, BMMSCs from patients with haemophilia have the potentials to proliferate and differentiate into chondrocytes *in vitro*, similarly with healthy adult. Therefore, it must be promising to treat the articular defects in patients with haemophilic arthropathy by the transplantation of autologous BMMSCs, as this procedure is safe and needs less invasive intervention.

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Disclosures

None of authors have any conflict of interest to disclose to the current manuscript.

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ETV6–NTRK3 as a therapeutic target of small molecule inhibitor PKC412

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ABSTRACT

The ETV6–NTRK3 (EN) fusion gene which encodes a chimeric tyrosine kinase was first identified by cloning of the t(12;15)(p13;q25) translocation in congenital fibrosarcoma (CFS). Since then, EN has been also found in congenital mesoblastic nephroma (CMN), secretory breast carcinoma (SBC) and acute myelogenous leukemia (AML). Using IMS-M2 and M0–91 cell lines harboring the EN fusion gene, and Ba/F3 cells stably transfected with EN, we demonstrated that PKC412, also known as midostaurin, is an inhibitor of EN. Inhibition of EN activity by PKC412 suppressed the activity of it downstream molecules leading to inhibition of cell proliferation and induction of apoptosis. Our data for the first time suggested that PKC412 could serve as therapeutic drug for treatment of patients with this fusion.

1. Introduction

The ETV6–NTRK3 (EN) fusion gene which encodes a chimeric tyrosine kinase was first identified by cloning of the t(12;15)(p13;q25) translocation in congenital (or infantile) fibrosarcoma, a mesenchymal malignancy of very young children [1]. Since then, EN has been also found in congenital mesoblastic nephroma [2], secretory breast carcinoma [3] and acute myelogenous leukemia (AML) [4].

ETV6 (also known as *TEL*) is an ETS family transcription factor thought to play a major role in early hematopoiesis and angiogenesis [5,6]. The *ETV6* gene has also been identified as a fusion partner in leukemia-associated chimeric proteins, such as *ETV6-PDGFR* [7], *ETV6-AML1* [8,9], *ETV6-JAK2* [10], *ETV6-ARG* [11], and others [12]. The *NTRK3* gene (also known as *TRKC*) encodes the transmembrane surface receptor for neurotrophin-3 involved in growth, development, and cell survival in the central nervous system [13].

In general, EN fusion transcripts encode the N-terminal pointed (PNT) domain of *ETV6* which is responsible for polymerization [14] fused to the C-terminal protein tyrosine kinase (PTK) domain of NTRK3. This fusion protein is similar in structure to other ETV6 chimeric PTKs [1]. Until now, two types of this fusion gene were found. The first one is detected in the non-hematological maglinancies in which the chimeric transcript encoded exon 1 to exon 5 of the *ETV6* gene fused to nucleotide (nt) 1741 of *NTRK3* gene. The second one is detected in leukemia in which the chimeric transcript encoded exon 1 to exon 4 of *ETV6* gene fused to nt 1741 of *NTRK3* gene.

In general, native *NTRK3* requires an extracellular ligand binding of neurotrophin 3 prior to its dimerization and autophosphorylation [15–19]. However, when fused to ETV6, the extracellular ligand binding domain of NTRK3 is abrogated and ETV6–NTRK3 bypassed this requirement, still keep itself autophosphorylation. Interestingly, in vitro and in vivo experiments have shown that the EN fusion protein has potential transforming activity in several cell lineages including fibroblast [20], hematopoietic cells [21], and breast epithelial cells [3].

The important role of EN in oncogenesis has been well known. However, specific treatment for patients expressing EN has not been achieved. Patients of congenital fibrosarcoma, congenital mesoblastic nephroma and secretory breast carcinoma have been considered as a good prognosis and rarely metastases [22–24]. Unfortunately, patients of leukemia seem to be poor prognosis. Two patients harboring EN have been reported no response to chemotherapy treatment suggesting it as a refractory leukemia [25,26]. Therefore, finding novel therapeutic treatment for leukemia patients harboring EN is extremely necessary.

We hypothesized that inhibition of EN could be an effective therapeutic strategy in treatment of patients harboring EN. In this report, we tested the potential therapeutic utility of the small molecule inhibitor PKC412 as an option for treatment of leukemias associated with EN fusion. PKC412 also known as midostaurin is the broad spectrum inhibitor of serine-threonine/tyrosine-protein kinases including protein kinase C (PKC), vascular endothelial growth factor receptor (VEGFR), fms-like tyrosine kinase (FLT3),

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platelet-derived growth factor receptor (PDGFR) and the stem cell factor, c-KIT [27,28]. Currently, PKC412 is used in phase IIB clinical trials for treatment of acute myeloblastic leukemia (AML) patients with FTL3 mutations [29] with minimal side-effect suggesting the utility of PKC412 for treatment.

2. Materials and methods

2.1. Plasmid construction

The construct of AML type of EN was described somewhere [30].

2.2. Cell lines, culture conditions and transfection

Experiments were conducted using two EN-positive AML cell lines: IMS-M2 and M0–91. IMS-M2 cell line was established from the bone marrow cells taken from a 59-year-old female with AML (FAB-M2), with chromosome abnormalities of 48,XX,add(6)(q27),+8, der(12)t(12;15)(p13;q25)inv(12)(p13;q15),der(15) t(12;15)(p13;q25), +der(15)t(12;15)(p13;q25), ETV6–NTRK3 fusion gene [4,25] and the type A *NPM1* mutation [31]. M0–91, an AML-M0 derived cell line, has recently been identified as a cell line expressing the ETV6–NTRK3 fusion gene by Gu et al. [32]. MOLM-13 and Jurkat cells were used as controls for evaluating PKC412 anti-proliferation effect.

All cell lines were grown in RPMI 1640 medium (Sigma–Aldrich, Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Nakalai Tesque, Kyoto, Japan) in a humidified incubator of 5% CO_2 at 37 °C. The Ba/F3 cells (ATCC, Manassas, VA, USA), maintained in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml of recombinant mouse interleukin 3 (IL-3; R&D Systems, Minneapolis, MN) were transfected with AML type of EN construct by lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected in medium containing mouse IL-3 and 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD, USA) for 2 weeks and subsequently subjected to limiting dilution to isolate single clones.

2.3. Reagents

PKC412, midostaurin was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO). Controlled cells were cultured with the same concentration of carrier DMSO as used in the highest dose of reagents. The concentration of DMSO was kept under 0.1% throughout all the experiments to avoid its cytotoxicity.

2.4. Cell proliferation assays

Proliferation was determined by trypan blue dye exclusion test. Cells in suspension were seeded in six-well plates at a density of 1×10^5 cells/ml in the presence of different concentrations of PKC412 for 3 days. In control wells, DMSO instead of PKC412 was added. After the treatment, 10 μl of the cell suspension was mixed with 10 μl of 0.4% trypan blue, and alive cells were counted manually using a hemacytometer. Results were calculated as the percentage of the values measured when cells were grown in the absence of the reagent. All experiments were performed in triplicate.

2.5. Western Blot analysis

The western Blot analysis was described in previous report [33]. Immunoprecipitation (IP) was performed as described previously [34]. Protein samples were electrophoresed through polyacrylamide gel and transferred to Hypond-P membrane (Amersham, Buckinghamshire, UK) by electro-blotting. After washing, the membrane was probed with following antibodies and antibody-binding was detected using enhanced chemiluminescence (ECL) (Amersham). The following antibodies were purchased from Cell Signaling Technology Japan (Tokyo, Japan): Phospho-p44/42 Map kinase (Thr202/Tyr204), phospho-Akt (Ser473), XIAP, Bcl-2, caspase-3, PARP, AKT, p44/42 MAPK, phospho-IKappaBalpha (Ser 32/36), and p-STAT5 (Y694). α-TrkC (C-14), STAT5 (C-17), and NFkB p52 (C-5), survivin (sc-17779), anti-rabbit IgG-HRP (sc-2317), and anti-mouse IgG-HRP (sc-2031) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-actin (A2066) was from Sigma–Aldrich.

2.6. Wright-Giemsa staining

For fragmented nuclei and condensed chromatin assessment, cells at a density of 1×10^5 cells/ml were treated with 100 nM PKC412. After 24 h incubation, cells were harvested and fixed onto slides by using a cytospin (Shandon, Shandon Southern Products Ltd., Cheshire, UK). Cells then were stained with Wright-Giemsa solution. Morphology of cells was observed under an inverted microscope.

2.7. DNA fragmentation assay

IMS-M2 cells were treated with or without 100 nM PKC412 for 24 h. Cells then were collected and total genomic DNA (gDNA) was extracted with a standard protocol. For DNA fragmentation assay, 10 μg gDNA of each sample was blotted and electrophoresed on 1.2% agarose gel. DNA fragmentation was observed under UV light.

2.8. Statistical analysis

All data were expressed as the mean ± standard deviation. Statistical analyses were done using Student's *t-test*, in which $p < 0.05$ was the minimum requirement for a statistically significant difference.

3. Results

3.1. PKC412 inhibits EN fusion tyrosine kinase in hematopoietic Ba/F3 cells

We evaluated the transforming property of EN in hematopoietic Ba/F3 cells. EN construct was stably transduced in Ba/F3 cells (Fig. 1A). Stable Ba/F3 cell line was assessed for IL-3 independent growth as a surrogate for transformation (Fig. 1B). EN effectively conferred IL-3 independence to Ba/F3 cells, whereas, Ba/F3 cells transduced with empty vector underwent apoptosis in the absence of IL-3.

To determine whether PKC412 inhibited EN activity, we have used the assay based on the work of Daley and Baltmore [35]. The transfected Ba/F3 cells were treated with or without 100 nM PKC412 for 72 h. Then cell proliferation assay was done to account for the inhibitory effect of reagent. 100 nM PKC412 significantly inhibited the cell proliferation of transfected Ba/F3 cells (Fig. 1C). To confirm whether the cell growth inhibition of PKC412 in transfected Ba/F3 cells is due to the lost of EN phosphorylation, we then checked the phosphorylation of EN in transfected Ba/F3 cells treated with or without 100 nM PKC412 for 8 h. As expected, the phosphorylation of this fusion protein was decreased by PKC412 treatment (Fig. 1D), suggesting that PKC412 inhibited the cell pro-

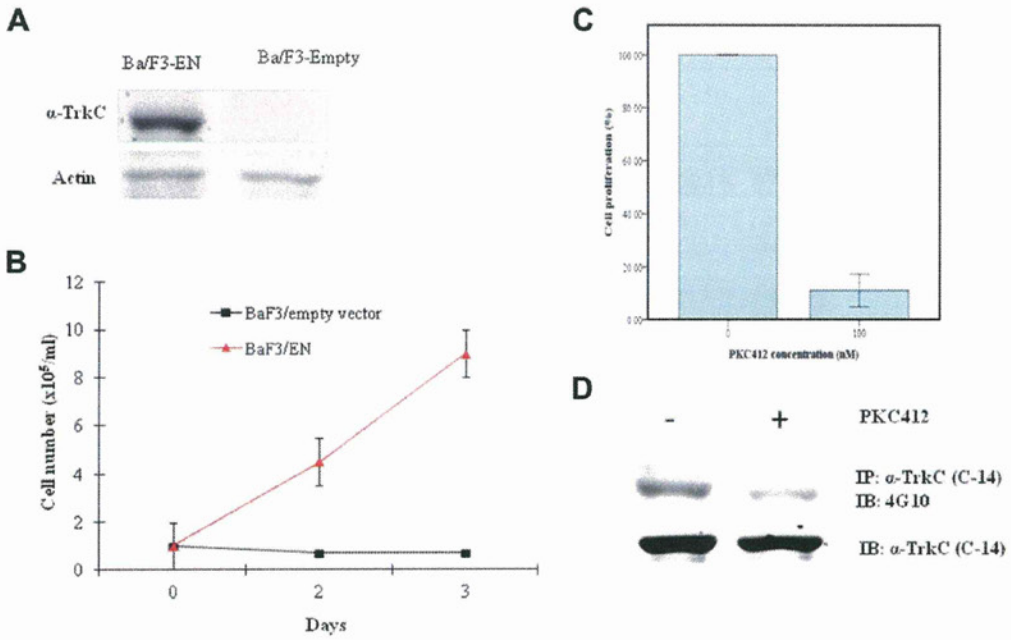

Fig. 1. PKC412 inhibits EN fusion tyrosine kinase in hematopoietic Ba/F3 cells. EN confers IL-3 independent growth to Ba/F3 cells. Successful transduction of EN construct (A) into Ba/F3 cells could induce IL-3 independent growth of Ba/F3 cells (B). 100 nM PKC treatment strongly inhibited the cell proliferation of EN-transfected Ba/F3 cells (C) and the activation of EN (D).

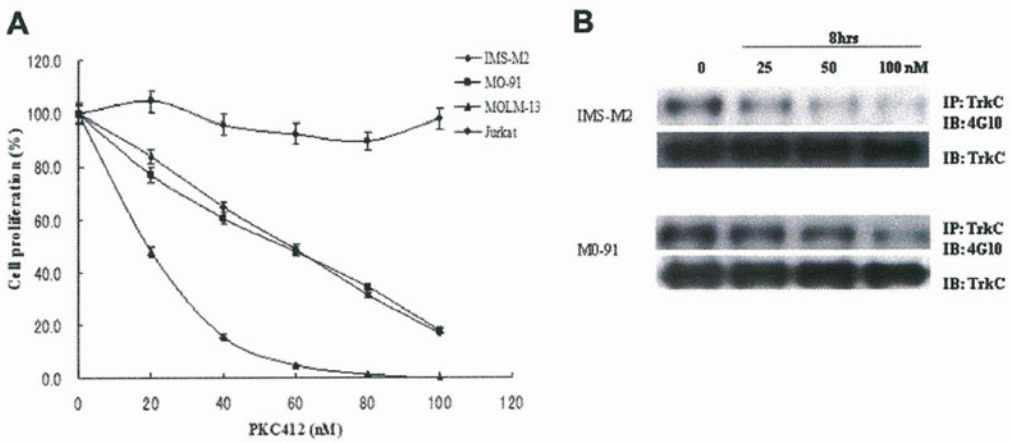

Fig. 2. Growth inhibition of EN-positive cell lines by PKC412. Panel A showed the anti-proliferation effect of PKC412 in cell lines harboring EN. Cell lines including IMS-M2, M0–91, MOLM-13 and Jurkat at a density of 1×10^5 cells/ml were treated with 10, 20, 40, 60, 80, 100 nM PKC412 or DMSO alone (0 nM PKC412) as control for 72 h. The number of alive cells was counted after trypan blue exclusion test. Results were calculated as the percentage of the control values. Panel B showed the effect of PKC412 on inhibition of EN activity in M0–91 and IMS-M2 cells after 8 h treatment.

liferation of transfected Ba/F3 cells by inhibiting the phosphorylation of EN. Altogether, the data demonstrate that EN could be a target of PKC412.

3.2. Growth inhibition of EN-positive cell lines by PKC412

Next we tested the inhibitory effect of PKC412 in a more clinically relevant system by performing a dose–response analysis using EN-positive cell lines including IMS-M2 and M0–91. Cell proliferation was evaluated using the trypan blue exclusion test and was confirmed by WST assay [36]. The growth of IMS-M2 and M0–91 cells was significantly inhibited in a dose-dependent manner by PKC412 treatment (Fig. 2A). However, the inhibitory effect of PKC412 obtained in MOLM-13 cells is more sensitive than in EN-positive cells. In contrast, the proliferation of EN-negative Jurkat cells seems to be not affected even at 100 nM PKC412 treatment (Fig. 2A).

To confirm whether PKC412 inhibited EN activity in EN-positive cell lines, we checked the phosphorylation status of EN in both two cell lines after 8 h treated with different concentration of PKC412. The results showed that PKC412 significantly inhibited EN phosphorylation in M0–91 and IMS-M2 cells in a dose-dependent manner (Fig. 2B). The data confirmed that EN is a target of PKC412.

3.3. Effect of PKC412 on AKT, MAPK, and STAT5 phosphorylation

To check whether PKC412 inhibits phosphorylation of *AKT*, *MAPK* and *STAT5*, we measure the phosphorylation status of these molecules after treated with 100 nM PKC412. The phosphorylation of the AKT, MAPK and STAT5 was significantly inhibited by PKC412

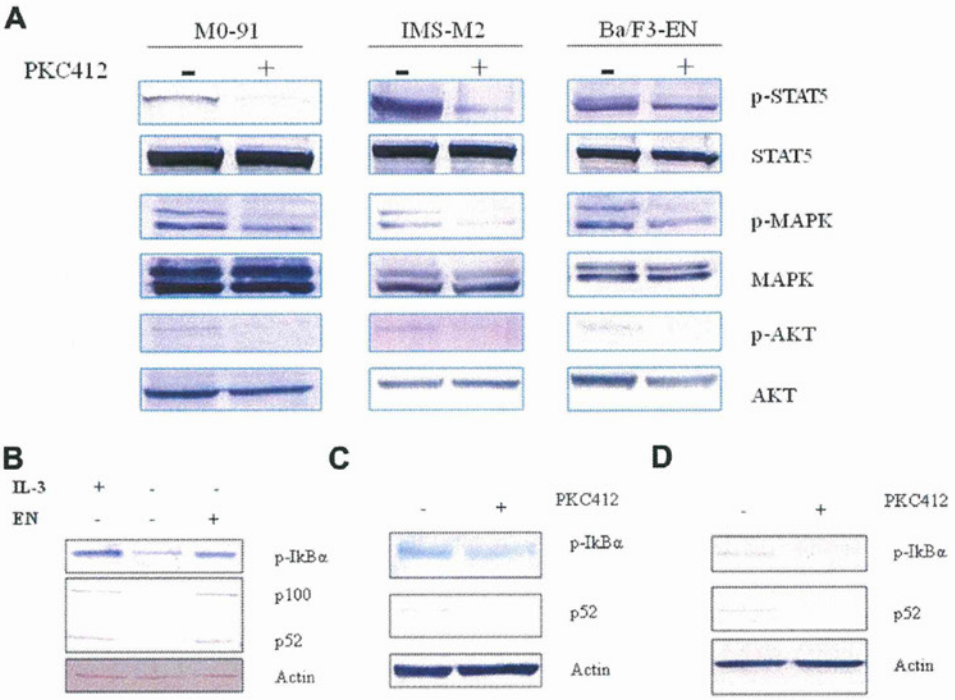

Fig. 3. Effect of PKC412 on EN-mediated signaling pathway Cells expressing EN have been treated with 100 nM PKC412 for 8 h. Total cell lysates were prepared and subjected to western blot analysis. Panel A showed that p-STAT5, p-AKT and p-MAPK were suppressed by PKC412 treatment. Panel B showed that EN could induce the activation of NF-κB pathway in EN-transfected Ba/F3 cells. PKC412 treatment could suppress the activation of p-IκBα and p52 in transfected Ba/F3 (C) and M0–91 cells (D).

(Fig. 3A). These results are consistent with previous reports that EN is the upstream molecule of Ras-MAPK and PI3K-AKT pathways [37].

3.4. PKC412 suppresses the activity of NFB

The EN-mediated signaling pathways controlling the cell proliferation and survival is not fully understood. In this study, we have shown that EN could induce the activation of NFκB. Introduction of EN construct into the Ba/F3 cells increases the phosphorylation of IκBα and the activation of p52 (Fig. 3B).

To test whether PKC412 treatment could affect on the activity of NFκB, M0–91 and Ba/F3 expressing EN cells were treated with PKC412 and subjected to western blot analysis. PKC412 treatment suppressed the phosphorylation of IκBα and the expression of p.52 in both EN-transfected Ba/F3 (Fig. 3C) and M0–91 (Fig. 3D) cells. In addition, treatment with DHMEQ, an inhibitor of NFκB, could suppress the proliferation of IMS-M2, M0–91 and EN-transfected Ba/F3 cells (data not shown). It suggests that NFκB pathway might also play an essential role in EN-inducing transformation.

3.5. PKC412 induces apoptosis in EN-positive cells

To determine whether PKC412 induces apoptosis in these cells, we have checked the appearance of some apoptotic markers in IMS-M2 after PKC412 treatment by western blot. As a result, 100 nM PKC412 treatment for 24 h induced the activation of caspase-3 (Fig. 4A, the top row) and subsequently inactive the activity of PARP (Fig. 4A, the bottom row). Moreover, PKC412 treatment showed the inhibition of survivin, XIAP and Bcl-2 expression (Fig. 4B). The PKC412-induced apoptosis is further supported by the morphology findings that PKC412 treatment show the DNA damage (Fig. 4C) and appearance of apoptotic bodies in IMS-M2 cells (Fig. 4D). Overall, the PKC412-induced cell death in IMS-M2 is apoptosis.

4. Discussion

Small molecule tyrosine kinase inhibitors, such as imatinib (Gleevec) or gefitinib (Iressa), are efficacious in treating certain human malignancies and solid tumors associated with dysregulation of tyrosine kinases. For example, imatinib has been successfully applied in clinical treatments of BCR-ABL-associated chronic myelogenous leukemia (CML), and activating mutations of c-KIT-associated gastrointestinal stromal cell tumors (GIST) [38,39]. In this report, we evaluated PKC412 as an inhibitor of activating EN. We observed that PKC412 effectively inhibits EN-dependent growth of Ba/F3 cells, as well as kinase activity and activation of downstream signaling effectors. Moreover, similar results were obtained in inhibition of EN-positive human cell lines by PKC412. The sensitivity to PKC412 is different among cell lines. MOLM-13 cells which harboring FLT-3 mutation showed more sensitive to PKC412 than EN-positive cell lines (Fig. 2A). It has been reported that PKC412 inhibits the growth of cancer cells with IC_{50} from 200–700 nM [40] comparing with IC_{50} obtained in EN-positive cell lines (60 nM) indicating that PKC412 could be a potential therapeutic reagent for treating this kind of cancers. However, in vivo experiments need to be done to test the effect of PKC412 on animal model.

The strength in the utility of PKC412 as a novel small molecule inhibitor in the treatment of EN-associated leukemia is that PKC412 is currently being evaluated in human clinical trials for treatment of AML associated with FLT3 with minimal effects, whereas no other EN inhibitors have been reported in the literature, to our knowledge, to be efficacious and well tolerated in patients. This brings much promise to patients who receive current highly aggressive therapies such as high-dose chemo or radio-ther-

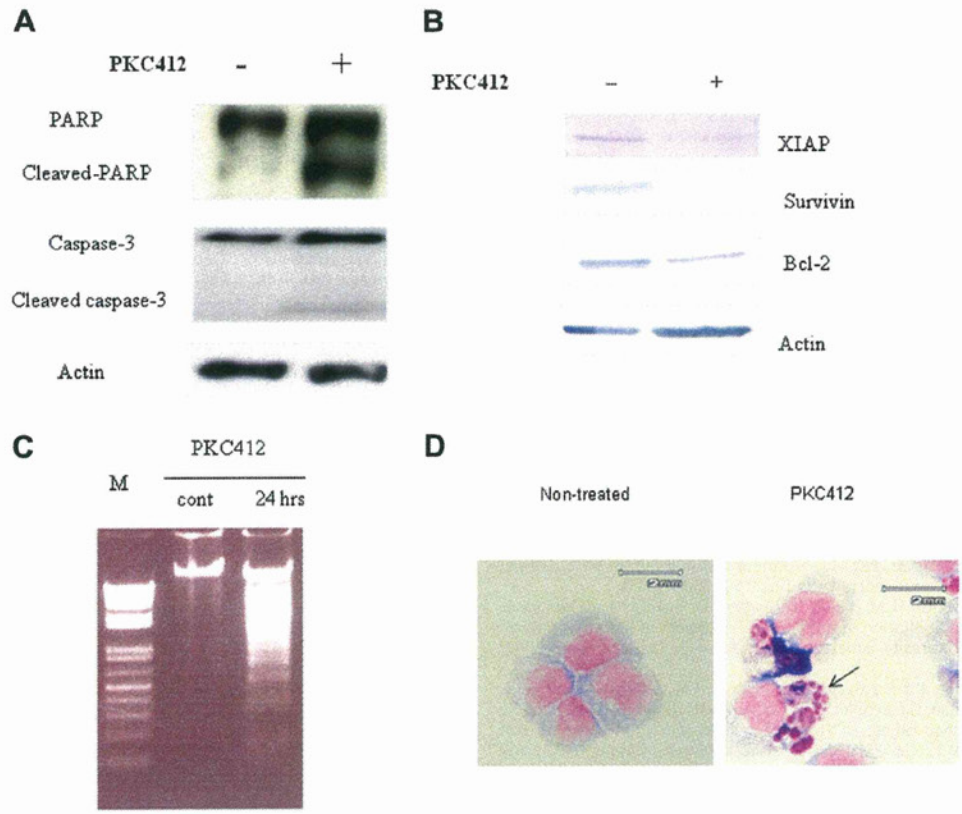

Fig. 4. PKC412 induces apoptosis in EN-positive cells IMS-M2 cells were treated with 100 nM PKC412 for 24 h. Cell lysates were subjected to western blot analysis with caspase-3 and PARP antibody. The results showed that treatment with PKC412 induced the cleavage of caspase-3 and PARP (A). 8 h treatment with PKC412 resulted in suppression of XIAP, survivin and Bcl-2 expression (B). Panel C showed the result of DNA fragmentation and panel D showed the morphological changes of IMS-M2 cells by PKC412 treatment. The arrow showed the appearance of apoptotic body in IMS-M2 cells after 24 h treating with PKC412.

apy followed by autologous transplantation of hematopoietic stem cells.

EN has also been identified in human congenital fibrosarcoma [1], congenital mesoblastic nephroma [2], and secretory breast carcinoma [3]. Therefore, our findings that the small molecule tyrosine inhibitor PKC412 inhibits EN activation may have therapeutic implications in the treatments of not only hematopoietic malignancies but also solid tumors associated with EN fusion.

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Brief Communication

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Acute Lymphoblastic Leukemia with t(1;19)(q23;p13)/TCF3-PBX1 Fusion in an Adult Male with Down Syndrome

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The chromosomal abnormality t(1;19)(q23;p13), leading to the production of the TCF3-PBX1 fusion transcript, is one of the common translocations in pediatric B precursor acute lymphoblastic leukemia (ALL) [1], but it is rarer in adults [2]. Although Down syndrome (DS) patients have a high risk of developing ALL in the first three decades of life [3], few reports have described adults with DS and ALL [4, 5]. We here report the first case of an adult patient with DS who was diagnosed as having ALL with t(1;19)(q23;p13)/TCF3-PBX1 fusion, complicated by coagulopathy and cerebral infarction.

A 28-year-old male with DS was admitted to our hospital because of high fever. He had no history of blood disorders or cardiovascular anomaly. Physical examination showed cervical lymphadenopathy of 2 cm in diameter. Hepatosplenomegaly was absent. Hemogram findings were as following: hemoglobin, 14.2 g/dl; white blood cell count, 16.9 × 10^9/l with 59% blasts, and platelet count, 66 × 10^9/l. The LDH value was 3,446 IU/l. The bone marrow examination revealed marked hypercellularity with 85% blasts, which were positive for CD10, CD19, cytoplasmic CD79a, HLA-DR, and TDT but negative for CD3, CD13, CD20, CD33, and CD34. Chromosomal analysis of bone marrow cells revealed 47, XY, +21 [6]/49, idem, t(1;19)(q23;p13.3), –5, –7, –13 [6]. Reverse transcription-polymerase chain reaction analysis confirmed the presence of the TCF3-PBX1 rearrangement. This patient was then diagnosed as having B-precursor ALL with t(1;19)(q23;p13)/TCF3-PBX1. Janus kinase 2 mutation was not detected. Central nervous system (CNS) involvement was absent. An induction chemotherapy regimen based on vincristine, prednisolone (PSL), cyclophosphamide, L-asparaginase (L-ASP), and pirarubicin was administered. The initial PSL response was poor. On day 14 of induction therapy, the coagulation test showed the following results: platelet count, 34 × 10^9/l; prothrombin time ratio, 1.18 (normal: 0.9–1.1); fibrinogen, 56 mg/dl (normal: 175–430 mg/dl); fibrin degradation products, 71 mg/l (normal: <4.0 mg/l); D-dimer, 50.5 mg/l FEU (normal: <0.5 mg/l FEU), and antithrombin III (AT III) activity level, 87% (normal: 80–120%). A diagnosis of disseminated intravascular coagulation (DIC) was made according to the diagnostic criteria of the International Society of Thrombosis and Hemostasis, and intravenous administration of recombinant thrombomodulin (rTM) was started. The administration of L-ASP on day

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15 was postponed. He recovered from the DIC after 6 days of the administration of rTM, but had clouding of consciousness and left-sided hemiparesis on day 20. Magnetic resonance imaging (MRI) on day 21 showed a subacute cerebral infarction of the right temporal lobe. Since AT III activity was low (37%) on day 21, we judged that the hypercoagulation status might induce thromboembolism. Then, anticoagulation therapy was begun, and his neurological symptoms, except mild left hemiparesis, were improved 10 days later. After that, he received MEC (mitoxantrone, etoposide, and cytarabine) [7] and hyper CVAD (cyclophosphamide, vincristine, Adriamycin, and dexamethasone) [8] treatment but did not achieve complete remission (CR). CNS involvement was observed 7 months after the initial diagnosis, and he died of the primary disease 2 months later.

Our experience has some implications for the treatment of ALL, especially with t(1;19)(q23;p13)/TCF3-PBX1, in adult DS patients. First, ALL with t(1;19) may be more resistant to chemotherapy in adult DS patients. Our patient did not achieve CR despite conventional chemotherapies equal to those for non-DS patients, although adult ALL with fusion of TCF3-PBX1 was reported to show a high CR rate but a short remission duration [2]. Since leukemic cells in our patient had an extraordinary karyotype in addition to trisomy 21 and t(1;19)(q23;p13), such as monosomy 7, there is a possibility that additional genetic abnormalities might make them more resistant. In any case, we need improvement in chemotherapy for ALL with TCF3-PBX1 fusion in DS patients. Second, we should pay more attention to thromboembolism in the treatment of ALL in adult DS patients, even though they have no cardiovascular anomaly. Although it has been shown that the incidence of macroangiopathy is low in adults with DS [9], several cases have been reported of thromboembolism developed in DS patients without cardiovascular anomaly [6, 10]. Together with these reports, the present case suggests that thromboembolism can occur in a hypercoagulated status in the treatment of ALL even in adults with DS.

This is the first report on ALL with t(1;19)(q23;p13)/TCF3-PBX1 in an adult with DS. The patient had a cerebral infarction during the induction therapy. He never achieved CR despite intensive chemotherapies, and died of the primary disease. Our experience suggests difficulty in the treatment of adult DS patients with ALL, especially with TCF3-PBX1 fusion. Therefore, we need improvement in chemotherapy, and careful supportive therapy for them.

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CORRESPONDENCE

Eltrombopag in Refractory Aplastic Anemia

TO THE EDITOR: As described by Olnes et al. (July 5 issue),[1] one possible concern with regard to eltrombopag therapy is that it stimulates c-MPL, which enhances clonal evolution to myelodysplasia or leukemia in patients with aplastic anemia. For example, the risk of progression from myelodysplastic syndromes to acute myeloid leukemia has been observed in a clinical trial of romiplostim, a c-MPL agonist.[2] Olnes et al. reported that as many as 3 of 25 patients with aplastic anemia (12.0% [95% confidence interval, 2.5 to 31.2]) had clonal evolution, including two cases of monosomy 7 and one case of myeloid leukemia. Since a 12-week observation period appeared to be too short for a new tumor to develop, treatment with eltrombopag might have enhanced the expanded clones that already existed before the treatment. Thus, clinical researchers who administer c-MPL agonists should carefully assess patients with aplastic anemia before treatment is begun. It should be noted that a conventional metaphase cytogenetic test and a cytomorphologic examination of bone marrow by light microscopy may not be sensitive enough to detect very minor clones. Could the authors share any techniques, such as newer cytogenetic detection methods, for detecting hidden malignant clones?[3]

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TO THE EDITOR: Olnes et al. report that treatment with eltrombopag led to clinically significant hematologic responses in 11 of 25 patients with severe aplastic anemia. However, in this trial involving patients who did not have a response to prior immunosuppressive therapy, 47 patients were screened for enrollment, 21 of whom were not enrolled. It is not clear from the study what made nearly half the screened patients ineligible. Did these patients have characteristics that made the authors think they would be less likely to have a response to eltrombopag? Because of the positive

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results of this study, would it now be appropriate to consider treating some of the patients who were not enrolled? In a few of the treated patients, it is possible that eltrombopag might have caused prolonged remissions. How do the authors explain this effect? Do they have data regarding the use of eltrombopag in combination with immunosuppression that show enhanced results in patients with aplastic anemia or in laboratory models?

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No potential conflict of interest relevant to this letter was reported.

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THE AUTHORS REPLY: Oshima and colleagues restate the concerns raised in our article. The risk of progression to myelodysplastic syndromes or acute myeloid leukemia may be particularly increased in patients with aplastic anemia that is refractory to immunosuppressive treatment. Although there are reports of progression to acute myeloid leukemia in patients with myelodysplastic syndromes who are treated with romiplostim, an alternative thrombopoietin agonist, there is as yet no evidence that the progression rate is higher than expected.[1] How best to monitor for preexisting or evolving clonal hematopoiesis in aplastic anemia is not clear. A limitation of any test is the profound hypocellularity of the marrow. There are no prospective trials comparing cytogenetic analysis with other methods. Cytogenetic detection can be unsuccessful in at least 10% of patients at presentation, and such analysis cannot detect minor clones. Fluorescence in situ hybridization is a more sensitive test,[2,3] but it screens for only a few abnormalities, and distinguishing a minor monosomy clone from background false positive nuclei is difficult. Array-based technologies such as single-nucleotide polymorphism (SNP) whole-genome arrays are exciting new approaches. Afable et al. reported copy-number abnormalities or loss of heterozygosity in 10 of 33 pretreatment samples with normal cytogenetic characteristics in patients with aplastic anemia.[4] Some abnormalities detected by SNP arrays disappeared on follow-up and may reflect nonpathogenic oligoclonal hematopoiesis. We are prospectively comparing metaphase cytogenetics with array-based methods to detect clonal populations in sequential bone marrow samples obtained from patients enrolled in ongoing trials of eltrombopag for the treatment of aplastic anemia.

In response to Akard: we excluded screened patients on the basis of protocol-defined criteria, most commonly elevated liver enzyme levels due to iron overload, dysplastic changes, or cytogenetic abnormalities on screening marrow examination, or because their blood counts were insufficiently abnormal for inclusion. The fact that a complete response was maintained in one patient after eltrombopag was discontinued was interesting and suggests that normalization of stem cells may persist once achieved, even in the absence of the drug. To further investigate this finding, we have begun to taper this drug in patients enrolled in our extension trial. We have initiated a trial of eltrombopag in combination with antithrombocyte globulin and cyclosporine in patients with severe aplastic anemia of new onset (ClinicalTrials.gov number, NCT01623167) and a trial of eltrombopag as a single agent in patients with moderate aplastic anemia (NCT01328587). However, until data are more abundant regarding clonal progression in patients with aplastic anemia who are treated with thrombopoietin agonists, we would continue to urge that all patients with bone marrow failure who are treated with eltrombopag be enrolled in clinical trials.

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Since publication of their article, the authors report no further potential conflict of interest.

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ORIGINAL ARTICLE

Association of hepatitis B with antirheumatic drugs: a case–control study

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Abstract
Background Though concern of hepatitis B virus (HBV) reactivation by antirheumatic agents has limited therapeutic opportunities in HBV-infected rheumatoid arthritis (RA) patients, the relative risks (RR) among such agents have not been clarified.
Objective We compared the reporting of antirheumatic-agent-associated hepatitis B.
Patients We assessed 92 hepatitis B cases and 98,069 controls from a population of 98,161 RA patients registered into the US Food and Drug Administration's (FDA's) adverse event database between 2004 and 2010.
Measurements A reporting odds ratio (ROR), a signal suggesting a risk for hepatitis B among antirheumatic agents, was measured.
Results Treatment with corticosteroids [ROR 2.3 (95 % confidence interval 1.3–4.0)], methotrexate [4.9 (3.9–6.0)], rituximab [7.2 (5.3–9.9)], tacrolimus [4.2 (1.5–11.9)], or reporting from Japan [2.2 (1.1–4.2)] were associated with higher signal, whereas adalimumab had a lower ROR [0.2 (0.1–0.4)].
Limitations There are known limitations of spontaneous reporting, such as underreporting, the Weber effect, reporting bias, indication bias, and limited clinical information such as HBV status.
Conclusions Adalimumab's low reporting rate is most likely be due to notoriety. However, the possibility that adalimumab might suppress reactivation of HBV cannot be denied. Until the possibility is clarified in well-designed clinical studies, physicians should use adalimumab cautiously in patients with HBV.

Keywords Hepatitis B · Rheumatoid arthritis · Antirheumatic drug · Adverse event reporting system (AERS) · Spontaneous report

Introduction

Progresses in pathophysiological knowledge, especially in cytokine cascades and their effector cells in rheumatoid arthritis (RA), have brought various developments of new antirheumatic agents. The classes of therapeutic agents directed against specific cytokines or effector cells in the disease process of RA, are: (1) disease-modifying antirheumatic drugs (DMARD), such as methotrexate (MTX), hydroxychloroquine (HCQ), leflunomide (LEF), and sulfasalazine (SSZ); (2) biological DMARDs, such as adalimumab (ADA), etanercept (ETA), infliximab (IFX), and rituximab (RTX); and (3) immunosuppressants, such as tacrolimus (TAC), azathioprine (AZT), cyclosporine (CSA), and mizoribine (MZB). These antirheumatic agents have greatly improved and expanded therapeutic options for RA. However, RA patients infected with hepatitis B virus (HBV) have been excluded from the benefit of therapeutic opportunities with these new agents. Reports about severe hepatitis case with increasing HBV-DNA after methotrexate (MTX) and corticosteroid therapy [1, 2]

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alerted the medical community about the use of antirheumatic agents in patients with HBV carrier status and proposed an algorithm for assessment and prevention of HBV reactivation in RA patients. The American College of Rheumatology (ACR) made recommendations on the use of DMARDs and biologics based on hepatitis type, Child–Pugh grade, and whether or not antiviral agents to treat hepatitis had been initiated. The college also asked physicians to consider the risks and benefits of all DMARDs [3]. The Japan College of Rheumatology (JCR) more strictly limited the use of tumor necrosis factor-alpha (TNF-α)-blocking biologics and MTX in HBV-carrying RA patients in 2008 (http://www.ryumachi-jp.com/english/index.html).

Serum hepatitis B surface antigen (HBsAg) is infrequent (0.1–0.5 %) in the normal population in the United States and western Europe. However, a prevalence of up to 5–20 % has been found in the Far East and in some tropical countries in patients with Hodgkin's disease, polyarteritis nodosa, and chronic renal disease [4]. Since HBV reactivation was reported not only in HBV carriers but also in RA patients with resolved or past HBV infection [5, 6], and the prevalence of concurrent and resolved HBV infection among RA patients in Japan was reported to be 0.8 % and 25.1 %, respectively [6], approximately one fourth of RA patients appear to be at risk for reactivation of HBV in Japan. As the reasons for restricting the use of certain DMARDs in HBV carriers by the colleges are mainly based on case series, case reports, and reviews of them, and the relative strength of risks still remain to be clarified. Recent reports suggest that screening for HBV infection and careful monitoring during the use of nonbiologic and biologic DMARDs may ameliorate the risk of severe hepatitis [5, 7, 8].

Guideline for the use of immunosuppressants and chemotherapy for malignant neoplasm in patients with HBV carriers is available in Japan [9]. The guidelines are not restricted to antirheumatic agents, but those who want to treat HBV-carrier RA patients may refer to the recommendations mentioned in the guidelines. The JCR also released recommendations regarding immunosuppressant use for RA patients with HBV infection in 2010 (http://www.ryumachi-jp.com/english/index.html). They describe HBV screening and the use of nucleoside analogs prior to immunosuppressive therapies. However, their recommendations lack a description regarding selection of antirheumatic agents. In Japan, one of the HBV-epidemic areas, no useful information appeared to be available for selecting antirheumatic agents for treating HBV-infected patients from the standpoint of relative risk (RR) for HBV reactivation.

There is no doubt that results from prospectively randomized clinical trial yield high-level evidence comparing risk of drugs. In order to assess risk level, prospective intervention studies using randomly assigned nonbiologic DMARDs or biologics to patients selected based on eligibility criteria and standardized assessment of occurrence of hepatitis are, of course, useful. However, ethical limitations and time/cost may make such a study unfeasible. Testing the risk of drugs with concern for severe adverse reactions may not be ethically acceptable. As clinical trials require exhaustive efforts and extensive costs and time, they may not provide timely information with a reasonable cost. Here, we propose the use of an adverse event reporting system (AERS) to rapidly estimate possible risks in these patients, as mentioned elsewhere [10, 11]. Despite limitations of the AERS, it may provide timely information with fewer costs [12, 13]. In this study, we compared reporting odds ratio (ROR) as a signal of risk for HBV reactivation associated with antirheumatic agents use. One can more effectively design clinical trials with less ethical concern to clarify crucial points based on the estimated results from AERS research.

Methods

Study design

A nested case–control analysis of antirheumatic-agent-associated HBV reported to the FDA between January 2004 and December 2010 was conducted. The subcohort study participants were individuals registered in the FDA AERS, with RA as an indication for drug use. Cases and controls were respectively defined as individuals with and without drug-associated HBV among the subcohort. The analyses included the number of unique cases and ROR among antirheumatic agents. In addition to monovariate analyses, a multivariate assessment by unconditional logistic regression was performed.

Datasource

The AERS database was downloaded from the FDA AERS Web page (http://www.fda.gov/), between first quarter 2004 and fourth quarter 2010.

Case identification

Drugs used to treat RA were identified as follows: First, the table for therapeutic indications was searched, which recorded an individual drug identifier (drug code) and corresponding indication for its use. As reported indications in the AERS database are coded according to the Medical Dictionary for Regulatory Activities (MedDRA) (Maintenance and Support Services Organization, Chantilly, VA, USA), we identified drugs with RA as their

indication by searching the preferred term (PT) coded as RHEUMATOID ARTHRITIS. Reports with drugs associated with RHEUMATOID ARTHRITIS as an indication of drug use were obtained from the AERS database. Search terms for drugs and events are provided in the electronic supplemental material (ESM). RTX is well known to induce reactivation of HBV in malignant lymphoma therapy. However, in this study, we analyzed RTX used only to treat RA. As the AERS database has some duplicate reports, we removed the older one from duplicate reports by sorting case identification numbers. After removing the duplicate report, there were 98,161 reports between 2004 and 2010. Then, the table for adverse reactions was searched for HBV, which is also coded by MedDRA PT. The term used for searching HBV is provided in the ESM. The database does not contain information for a more detailed discussion regarding screening for HBV prior to treatment or antibody/antigen status, such as HBsAg, hepatitis B core antibody (HBcAb), or DNA replication. Thus, the cases identified may possibly include HBV reactivated from HBsAg carriers or past/resolved HBV infection (occult HBV infection). We intended to use controls to obtain background information for disproportionality analysis in demographic data or drug usage. Thus, we did not select controls matched for age, gender, reporting countries, and so on.

Analysis

The identified reports were tabulated by reporting year, gender, age, and drugs. Reporting year is calculated from the date on which the FDA received the report. Demographic data were compared, and P value calculated with Fisher's exact test. Drug-associated HBV was tabulated and the ROR calculated. Drugs associated with HBV suggested by the monovariate analyses and with five or more cases were further analyzed by an unconditional logistic regression model.

Software

Microsoft Access 2003 and Excel (Microsoft Corporation, Redmond, WA, USA) were used for data management and basic tabulations. Logistic regression was performed using CDC EpiInfo software [Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA].

Results

There were 98,161 AE reports associated antirheumatic-agent use. Among them there were 92 HBV cases and 98,069 controls (Table 1). Age [mean (SD) years] for cases and controls were 62.9 (10.3) and 60.3 (43.7), respectively. Differences did not appear to be meaningful. The proportion of male case ($n = 27$, 29.3 %) appeared higher than for controls ($n = 17{,}218$, 17.6 %). Number of reports per year appeared to increase over the study period for both cases and controls, without notable differences between them. Case reports from Japan [19 (20.7 %)] were remarkably higher than for controls [3,742 (3.6 %)]. Fifty-three cases (57.6 %) among 92 and 83,846 (85.5 %) among controls were treated with a single agent. Conversely, 20 (21.7 %) and 19 (20.7 %) cases were proportionately higher ($P < 0.001$) than 8,770 (8.9 %) and 5,453 (5.6 %) controls treated with two and three or more antirheumatic agents, respectively (Table 1). ROR for each antirheumatic agent are shown in Table 2. Drug categories, such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, DMARDs, and immunosuppressants had higher lower limit of 95 % confidence intervals (CI) for ROR than 1.0. Individual drugs, such as HCQ, MTX, SSZ, RTX, CSA, cyclophosphamide (CP), and TAC indicated high RORs. However, among them, case numbers for HCQ, SSZ, CSA, and CP appeared too limited to assess drug signals. Interestingly, RORs were not high for TNF-α-blocking biologics, such as ADA, ETA, and IFX (Table 2).

Six (6.5 %) of 92 HBV cases were reported as fulminant hepatitis. Five (83.8 %) of the six fulminant hepatitis cases resulted in fatal outcome; four (66.7 %) of six were treated with MTX. Twenty-seven (29.3 %) of 92 HBV cases resulted in fatal outcome; methotrexate was most frequently used in 20 of the 27 fatal cases.

Logistic regression model was built using variables that significantly influenced HBV indicated by monovariate analyses (Table 3). Factors that increased HBV reporting were drugs such as corticosteroids, MTX, RTX, TAC and reports from Japan. On the other hand, ADA was associated with low ROR (Table 3). There was an interaction between ADA and Japan: of six ADA-associated HBV cases, thre were reported from Japan. These three cases were reported to the FDA in 2010. As there was a risk of HBV communication noted in the package insert for ADA in Japan in 2010, the three case reports could be influenced by a notoriety bias [14]. Moreover, as the number of ADA-associated HBV was very limited, we feel the interaction was not clinically meaningful. Whereas no interaction in combination with antirheumatic agents was found, we conducted another analysis by case/noncase methodology to assess effects of concomitant use of two or more of corticosteroids: HCQ, MTX, RTX, and TAC. There were 4,598 patients concomitantly treated with two or more of the indicated drugs, and 24 of them were reported as having HBV [ROR 7.2, (CI 5.7–9.2)].

HBV reactivation following immunosuppressive therapy is clinically important for several reasons. First, it can

Table 1 Baseline characteristics of all individual case safety reports for antirheumatic drugs in the US Food and Drug Administration Adverse Events Reporting System (FDA AERS) 2004–2010

Variable	Individual case safety reports (n = 98,161)				Crude odds ratio	P value*
	Hepatitis B case		Other AE control			
	(n = 92)		(n = 98,069)		(95 % confidence interval)	
Patient age mean (SD), years[a]	62.9	(10.3)	60.3	(43.7)		
Age missing	49	53.3	68,612	70.0	0.49 (0.32–0.75)	<0.001
Female sex	58	63.0	68,721	70.1	0.73 (0.47–1.15)	0.140
Male sex	27	29.3	17,218	17.6	1.95 (1.20–3.10)	0.006
Sex missing	7	7.6	12,130	12.4	0.58 (0.23–1.26)	0.204
Reporter						
Health care professional	71	77.2	38,086	38.8	5.32 (3.23–9.13)	<0.001
Non-health-care professional	13	14.1	45,021	45.9	0.19 (0.10–0.35)	<0.001
Reporter missing	8	8.7	14,962	15.3	0.53 (0.22–1.09)	0.082
Reporting year						
2004	8	8.7	9,736	9.9	0.86 (0.36–1.78)	0.861
2005	9	9.8	8,735	8.9	1.11 (0.49–2.21)	0.714
2006	6	6.5	8,886	9.1	0.70 (0.25–1.59)	0.583
2007	8	8.7	14,615	14.9	0.54 (0.23—1.12)	0.107
2008	19	20.7	16,671	17.0	1.27 (0.72–2.13)	0.333
2009	15	16.3	18,012	18.4	0.87 (0.46–1.52)	0.687
2010	27	29.3	21,414	21.8	1.49 (0.91–2.36)	0.099
Reporter country						
United states	34	37.0	60,966	62.2	0.36 (0.23–0.55)	<0.001
Japan	19	20.7	3,742	3.8	6.56 (3.73–11.00)	<0.001
United Kingdom	8	8.7	5,474	5.6	1.61 (0.67–3.32)	0.175
Other	18	19.6	12,957	13.2	1.60 (0.90–2.70)	0.088
Reporter country missing	13	14.1	14,930	15.2	0.92 (0.47–1.66)	0.885
Therapy						
Monotherapy	53	57.6	83,846	85.5	0.23 (0.15–0.36)	<0.001
NSAIDs	3	3.3	2,175	2.2	1.49 (0.30–4.49)	0.461
DMARDs	9	9.8	3,359	3.4	3.06 (1.35–6.10)	0.004
Biologics	40	43.5	77,628	79.2	0.20 (0.13–0.31)	<0.001
Immunosuppressants	1	1.1	238	0.2	4.52 (0.11–26.06)	0.201
Corticosteroids	0	0.0	446	0.5	0.00 (0.00–8.99)	1.000
Dual Therapy	20	21.7	8,770	8.9	2.83 (1.63–4.70)	<0.001
Three or more antiRheumatic agets	19	20.7	5,453	5.6	4.42 (2.52–7.41)	<0.001

SD standard deviation, *NSAIDs* nonsteroidal anti-inflammatory drugs, *DMARDs* disease-modifying antirheumatic drugs

* P value is not intended to test statistical differences between two groups but to highlight the differences

[a] All data except patient age are shown as number (%)

progress to fatal hepatitis, even though the frequency is rare. Second, it is preventable by proper management. Thus, we assessed 27 HBV cases with fatal outcome (Table 4). The time period between the beginning of primary suspected drug initiation and HBV onset was >2 months; more than half of the reports were from East Asia.

Possible factors that may suppress HBV reporting involving ADA among the three TNF-α-blocking biologics are shown in Table 5. Comparison of the age, gender, and concomitant medication, such as MTX and corticosteroids, which are associated with higher HBV reporting, may not explain the low ROR for ADA.

Discussion

We found several factors, such as treatment with corticosteroids, MTX, RTX, TAC, and reporting from Japan associated with a higher prevalence of HBV, and the use of

Table 2 Reported hepatitis B cases

Drug	Adverse events		Proportion of hepatitis B (%)	Reporting odds ratio	*P* value*
	Hepatitis B	Other AE		(95 % confidence interval)	
NSAIDs	11	5,642	0.19	2.2 (1.6–3.0)	0.021
Corticosteroids	27	7,315	0.37	5.1 (4.0–6.4)	<0.001
DMARDs	41	14,930	0.27	4.4 (3.6–5.5)	<0.001
Actarit	0	43			
Auranofin	0	30			
Bucillamine	0	285			
Chloroquine	0	94			
Gold	0	45			
Hydroxychloroquine	4	1,134	0.35	3.8 (2.3–6.4)	0.023
Leflunomide	4	3,198	0.12	1.3 (0.8–2.2)	0.547
Lobenzarit	0	6			
Methotrexate	35	10,896	0.32	4.9 (3.9–6.0)	<0.001
Minocycline	0	37			
Penicillamine	0	62			
Sulfasalazine	4	1,340	0.30	3.2 (1.9–5.4)	0.038
Biologics	73	90,235	0.08	0.3 (0.2–0.4)	<0.001
Abatacept	2	1,585	0.13	1.3 (0.6–2.7)	0.662
Adalimumab	6	29,326	0.02	0.1 (0.1–0.2)	<0.001
Anakinra	0	512	0.00		
Etanercept	38	43,788	0.09	0.8 (0.7–1.0)	0.531
Infliximab	15	13,119	0.11	1.2 (0.9–1.6)	0.442
Rituximab	12	1,976	0.60	7.2 (5.3–9.9)	<0.001
Tocililzumab	1	868	0.12	1.2 (0.4–3.3)	0.559
Immunosuppressants	8	949	0.84	9.7 (6.7–14.1)	<0.001
Azathioprine	0	289			
Ciclosporin	2	178	1.11	12.2 (5.9–25.0)	0.013
Cyclophosphamide	1	46	2.13	23.4 (8.4–64.6)	0.043
Mizoribine	0	75			
Mycophenolate	0	16			
Mycophenolic acid	0	1			
Prednimustine	0	1			
Sirolimus	0	1			
Tacrolimus	5	367	1.34	15.2 (9.6–24.3)	<0.001
Temsirolimus	0	6			

NSAIDs nonsteroidal anti-inflammatory drugs, *DMARDs* disease-modifying antirheumatic drugs

* *P* value is not intended to test statistical differences between two groups but to highlight the differences

ADA was associated with a low ROR for HBV. Three billion individuals worldwide are reported to be exposed to HBV. Prevalence of HBV infection in the population of the area of focus in this study will affect the number of reports. The Far East and the tropics of Asia are epidemic for HBV [15]. As Japan is the most frequently reporting country of AEs to the FDA within the epidemic area, Japan reported a relatively large number of HBV cases. Although use of MTX and biologics in HBV has been simply contraindicated by the JCR (http://www.ryumachi-jp.com/english/index.html), approximately one forth of RA cases were reported to be infected with HBV in Japan [6]. Reactivation of HBV occurs not only in individuals who are HBsAg-positive [16] but also in HBsAg-negative individuals with occult HBV infection [17]. Thus, discussion of their treatment with antirheumatic agents, such as nonbiologic DMARD, biologics, and immunosuppressants, must not be avoided.

We did not select matched controls from a subcohort population according to factors such as gender, age, and reporting countries in this analysis, mainly for two reasons: First, our aim was to identify factors among any potential risk

Table 3 Logistic regression for hepatitis B

Variables	Odds ratio (95 % confidence interval)		*P* value
Adalimumab (yes/no)	0.2	(0.1–0.4)	<0.001
Corticosteroids (yes/no)	2.3	(1.3–4.0)	0.004
Methotrexate (yes/no)	3.1	(1.9–5.2)	<0.001
NSAIDs (yes/no)	0.8	(0.4–1.5)	0.448
Rituximab (yes/no)	5.4	(2.9–10.2)	<0.001
Tacrolimus (yes/no)	4.2	(1.5–11.9)	0.006
Gender (M/F)	1.6	(1.0–2.5)	0.058
Gender (missing/F)	0.8	(0.4–1.8)	0.573
Japan (yes/no)	2.2	(1.1–4.2)	0.024

NSAIDs nonsteroidal anti-inflammatory drugs

factors, including age, gender, or reporting countries. Second, as numbers of reports within the database vary among drugs, selecting matched controls might eliminate drugs with small number of reports; consequently identification of these agents would not be assessed. Instead, we tabulated ROR, which is designed to identify potential signals of risk based on disproportionality. ROR is widely used for detecting signals for medical products, which can be regarded as relative risk [18]. As we describe under the "Limitation" section, there are reporting and therapeutic biases in FDA AERS. In this study, we selected cases and controls out of the biased population; thus, the effect of the reporting and therapeutic biases may in part be ameliorated.

It has been reported that reactivation of HBV in HBV carriers occurred and sometimes resulted in fatal or life-threatening hepatitis following chemotherapies for malignant neoplasms or immunosuppressive treatment for organ transplantations. One mechanism proposed was that corticosteroids stimulate replication of the virus, as there is a glucocorticoid enhancement element in the HBV gene [16, 19]. Actually, the use of corticosteroids in chemotherapeutic regimens is linked to increased risk of HBV reactivation [20]. In our analyses, use of corticosteroids indicated an independent signal for HBV (Table 3). In Japan, use of DMARDs, such as MTX or other low molecular weight DMARDs and biologics, is permitted in well-equipped hospitals only and by physicians whose knowledge and experience include the use of DMARD therapy, because of the possibility of serious toxic reactions that can be fatal. Conversely, corticosteroids may be prescribed by general physicians due to the various indications for their use. Thus, corticosteroids are widely used to treat RA in Japan. As our analyses indicated a high indication for HBV following corticosteroid use, those who treat RA with corticosteroids must carefully screen patients for HBV infection and follow the recommendation of the JCR regarding HBV infection.

As the ROR value for MTX is high, the concern of the JCR that led to restricted its use for HBV carriers appears to be valid (http://www.ryumachi-jp.com/info/guideline_mtx.html) (Table 3). On the other hand, the ROR value for LEF and anti-TNF-α biologics such as ETA and IFX, were not high (Table 2). Perhaps, rather, we should describe TNF-α-blocking biologics as appearing to be associated with low ROR (Table 2). Actually, ADA was associated with low ROR, even in multivariate analysis (Table 3). HBV is a nonlytic virus, which does not directly cause cytolysis of infected hepatocytes [21]. Liver damage and viral clearance after an HBV infection are thought to be mediated by the host's immune response to viral antigens [22]. As TNF-α-blocking biologics also have an immunosuppressive mechanism, we feel that TNF-α-blockers do not suppress HBV reactivation. However, the specific blockage of TNF-α cascade may possibly have less affect on viral reactivation than other DMARDs, which are thought to suppress broad immune responses. Previous animal experiments using hepatitis-model mice and HBsAg-specific cytotoxic T-cell clones reported that monoclonal antibody to TNF-α was protective against cytotoxic T-cell-induced liver damage [23]. Thus, TNF-α was thought to play a role in cytotoxic response to HBV-antigen-positive hepatocytes. Also, suppressing TNF-α signaling is thought to suppress the occurrence of HBV, even in cases with increased viral replication. Actually, a previous report described a relevant observation of a case with increased HBV DNA replication without increased transaminase level after a TNF-α-blocking therapy in combination with other DMARDs [6].

We further explored the possible factors that suppressed HBV reporting in ADA among the three TNF-α-blocking biologics. We compared age, gender, and concomitant medication, such as MTX and corticosteroid, that are associated with higher HBV reporting (Table 5). As the number of other AEs is significant, demographic data for other AEs in each biologic category may reflect those of a population treated with the indexed drug. However, this tabulation did not indicate clear factors that suppressed ADA reporting. In the HBV-infected liver, TNF-α is thought to be involved both in viral clearance and hepatocyte damage. The former activity appears to suppress clinical development of viral hepatitis, whereas the latter activity appears to enhance it. Moreover, TNF-α induces pleiotropic cellular responses, such as cell proliferation, production of various cytokines, and cell death. Thus, TNF-α appears to cause complicated immune responses. Therefore, fine differences in TNF-α-blocking activities among different TNF-α-blocking agents could induce incomprehensibly different host responses.

Although there is no conclusive explanation for the observed differences in reporting of viral hepatitis among