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Intracellular galectin-9 activates inflammatory cytokines in monocytes

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Whether galectin-9 plays a role in inflammatory responses remains elusive. The present study was designed to determine the role of intracellular galectin-9 in activation of inflammatory cytokine genes in human monocytes. Galectin-9 expression vector pBKCMV3-G9 was transiently co-transfected into THP-1 monocytic cells along with luciferase reporters carrying gene promoters of IL-1α (*IL1A*), IL-1β (*IL1B*) and *IFNγ*. Transient transfection studies showed that galectin-9 over-expression activated all three gene promoters, suggesting that intracellular galectin-9 induces inflammatory cytokine genes in monocytes. Galectin-9 over-expression also activated NF-IL6 (C/EBP β) and AP-1, but not NF-κB. In contrast, extracellular galectin-9 is not involved in regulation of inflammatory cytokines. Immunoprecipitation/Western blotting, using anti-galectin-9 Ab and anti-NF-IL6 Ab, showed physical association of intracellular galectin-9 with NF-IL6. RT-PCR confirmed that galectin-9 over-expression increased IL-1α and IL-1β mRNA levels in THP-1 cells. The interaction of galectin-9 with NF-IL6 was enhanced following LPS treatment in THP-1 cells. Intracellular galectin-9 synergized with LPS to activate NF-IL6. Nuclear translocation of galectin-9 was also observed in THP-1 cells treated with LPS. Our results indicate that galectin-9 is a LPS-responsive factor, and further demonstrate that intracellular galectin-9 transactivates inflammatory cytokine genes in monocytes through direct physical interaction with NF-IL6.

Introduction

Galectins are a protein family of animal lectins that modulate various extracellular (by interacting with cell surface and extracellular matrix glycoproteins and glycolipids) and intracellular (by interacting with cytoplasmic and nuclear proteins) signaling pathways. To date, 15 galectins have been cloned in mammals (Barondes *et al.* 1994a). Galectins exhibit affinity for β-galactosides, which share certain conserved sequence elements (Barondes *et al.* 1994b), and play modulatory roles in diverse biological processes such as cell adhesion, cell proliferation (Perillo *et al.* 1998; Asakura *et al.* 2002; Nishi *et al.* 2003; Zick *et al.* 2004), cell apoptosis (Perillo *et al.* 1995; Kashio *et al.* 2003), chemoattraction and immunomodulation of inflammation (Liu 2000; Rabinovich *et al.* 2002; Almkvist & Karlsson 2004). Galectins can act as inflammatory mediators, and are involved in the recruitment of polymorphonuclear leukocytes from the blood stream and cross-link of polymorphonuclear leukocytes with the endothelium (Almkvist & Karlsson 2004).

Members of the galectin family are structurally classified into three groups. Galectin-1, 2, 7, 10 and 13 are prototype galectins, whereas galectin-3 is a chimera type. In contrast, galectin-4, *8*, 9 and 12 belong to the tandem repeat type subfamily, which is characterized structurally by the presence of two distinct carbohydrate recognition domains, with different sugar binding specificities, joined by a linker peptide. Galectin-9 exhibits various biological activities such as chemoattraction, cell aggregation, induction of superoxide production and prolongation of cell survival of eosinophils (Matsumoto *et al.* 2002). In addition, galectin-9 can function as a proapoptotic factor in a variety of cells, including activated T lymphocytes (Kashio *et al.* 2003), thymocytes and tumor cells (Kageshita

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et al. 2002). Galectin-9 is also a potential prognostic factor as it exhibits antimetastatic properties in breast cancer (Irie *et al.* 2005). Galectin-9 is expressed in activated T cells, mouse embryonic kidney, and tissues of patients with Hodgkin's disease, monocytes/macrophages, Jurkat, THP-1, and RPMI-8866 cells (Spitzenberger *et al.* 2001). In mice, this factor is widely distributed, including the liver, small intestine, thymus, kidney, spleen and lung. Phorbol 12-myriastate 13-acetate up-regulates galectin-9 production in Jurkat cells (Chabot *et al.* 2002). Furthermore, matrix metalloproteinase and protein kinase C are involved in the release of galectin-9 from Jurkat cells (Chabot *et al.* 2002). Human endothelial cells treated with interferon (IFN)-γ exhibit increased production of galectin-9 (Imaizumi *et al.* 2002). Lipopolysaccharide (LPS) enhances the expression levels of galectin-9 mRNA and protein in a time-dependent manner (Kasamatsu *et al.* 2005). The present study was designed to determine the significance of galectin-9 in the inflammatory processes. The results demonstrated that intracellular galectin-9 functions as a transcriptional activator of inflammatory cytokine genes in monocytes/macrophages.

Results

Galectin-9 induces expression of IL-1α and IL-1β mRNAs in THP-1 monocytic cells

Galectin-9 is expressed in various types of cells such as T-cells, fibroblasts, endothelial cells, mast cells, macrophages, astrocytes and eosinophils. In the present study, mRNA expression of inflammatory cytokines such as IL-1α and IL-1β in THP-1 cells transiently transfected with galectin-9 expression vectors was examined by using RT-PCR. Three kinds of galectin-9 expression vectors, pBKCMV3-G9(S), pBKCMV3-G9(M) and pBKCMV3-G9(L) were used. Three isoforms of galectin-9 that differed only in the length of their linker peptide region have been identified (Hirashima 2000). The short-sized isoform of galectin-9, galectin-9(S) was found to have a linker peptide region of 14 amino acids, whereas the medium and the long-sized isoforms of galectin-9, galectin-9(M) and galectin-9(L) have a linker peptide region of 26 and 58 amino acids, respectively. The expression vectors for galectin-9(S), galectin-9(M) and galectin-9(L) were pBKCMV3-G9(S), pBKCMV3-G9(M) and pBKCMV3-G9(L), respectively. THP-1 cells that did not over-express galectin-9 showed no significant expression of IL-1α or IL-1β mRNA (Fig. 1A,B). Transient transfection of either pBKCMV3-G9(S), pBKCMV3-G9(M) or pBKCMV3-G9(L) into THP-1 cells induced both IL-1α and IL-1β mRNA expression

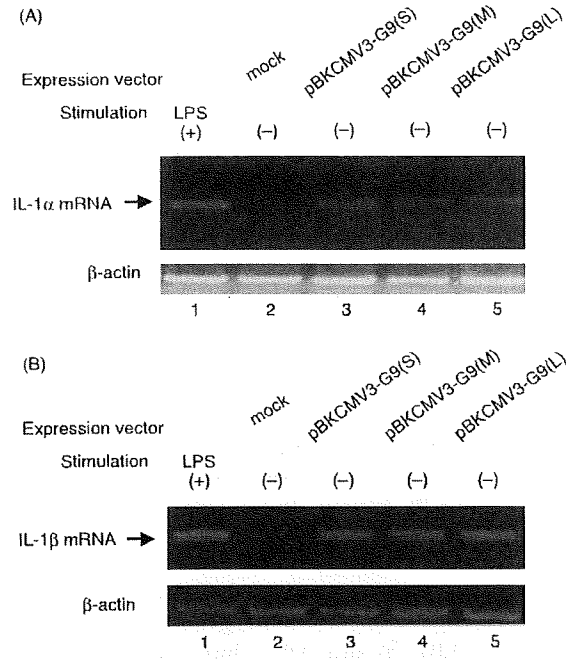

Figure 1 Expression of IL-1α and IL-1β mRNAs in THP-1 cells transfected with galectin-9 expression vectors. Three micro grams of pBKCMV3-G9(S), pBKCMV3-G9(M) or pBKCMV3-G9(L) were transiently transfected into THP-1 cells, and 24 h after transfection, expression of IL-1α (A) and IL-1β (B) mRNAs was examined by RT-PCR. RT-PCR was carried out as described in Experimental Procedures section. As a positive control, THP-1 cells were treated with LPS. Representative results of three experiments with similar findings.

(Fig. 1A,B). These results indicate that galectin-9 enhances the mRNA expression of IL-1α and IL-1β.

Effects of galectin-9 on IL1A, IL1B and IFNγ promoter activities in THP-1 cells

To examine the effects of galectin-9 on the transcriptional regulation of inflammatory cytokine genes, pBKCMV3-G9(S), pBKCMV3-G9(M) or pBKCMV3-G9(L) was co-transfected into THP-1 cells along with pGL3IL1α reporter for *IL1A* gene promoter. Over-expression of each of the three isoforms of galectin-9 resulted in similar enhancement of *IL1A* promoter activity (Fig. 2). Furthermore, transfection of pBKCMV3-G9(S) dose-dependently induced promoter activities of *IL1B* and *IFNγ* as well as *IL1A* (Fig. 3A–C). Transfection of pBKCMV3-G9(S) at 1.5 μg resulted in approximately threefold, eightfold and eightfold increase in activities of *IL1A*, *IL1B* and *IFNγ* promoters. In contrast, no activation of *IL1B* promoter was observed, when galectin-8 expression vector

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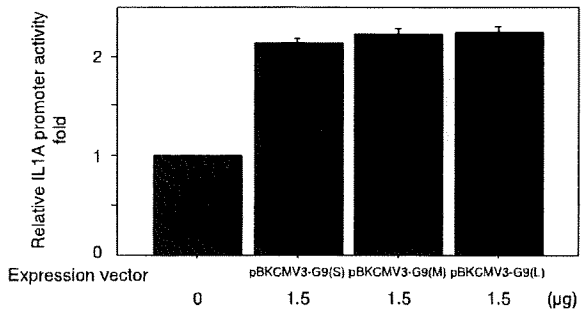

Figure 2 Effects of galectin-9(S), (M) and (L) on *IL1A* promoter activity in THP-1 cells. An amount of 1.5 μg of pBKCMV3-G9(S), pBKCMV3-G9(M) or pBKCMV3-G9(L) were transiently transfected into THP-1 cells, together with 1.0 μg of *IL1A* promoter reporter. The total amount of transfected DNA was kept constant (3.0 μg) by adding control vector. Data are mean ± SD of triplicate samples. Luciferase assays were carried out as described in Experimental Procedures. Data were normalized by internal control Renilla luciferase activity.

pBKCMV3-G8 was used instead of pBKCMV3-G9 (Fig. 3D).

In contrast to the data obtained from transient transfection studies using pBKCMV3-G9(S), incubation of THP-1 cells carrying *IL1A* promoter reporter pGL3IL1α with exogenous galectin-9 protein, hG9NC (null) 100 nM for 24 h resulted in inhibition of *IL1A* promoter activity. Furthermore, the galectin-9-induced inhibition of *IL1A* promoter activity was prevented by lactose (30 mM), but not sucrose (30 mM) (Fig. 4). The most biological effects of extracellular galectins are mediated by their carbohydrate-binding activity. These data indicate that unlike intracellular galectin-9, extracellular galectin-9 is not involved in activation of inflammatory cytokine gene promoters.

Effects of galectin-9 on transcription factors NF-IL6, NF-κB and AP-1 in THP-1 cells

Induction of genes of inflammatory cytokines such as *IL1A* and *IL1B* and *IFNγ* involves binding of several

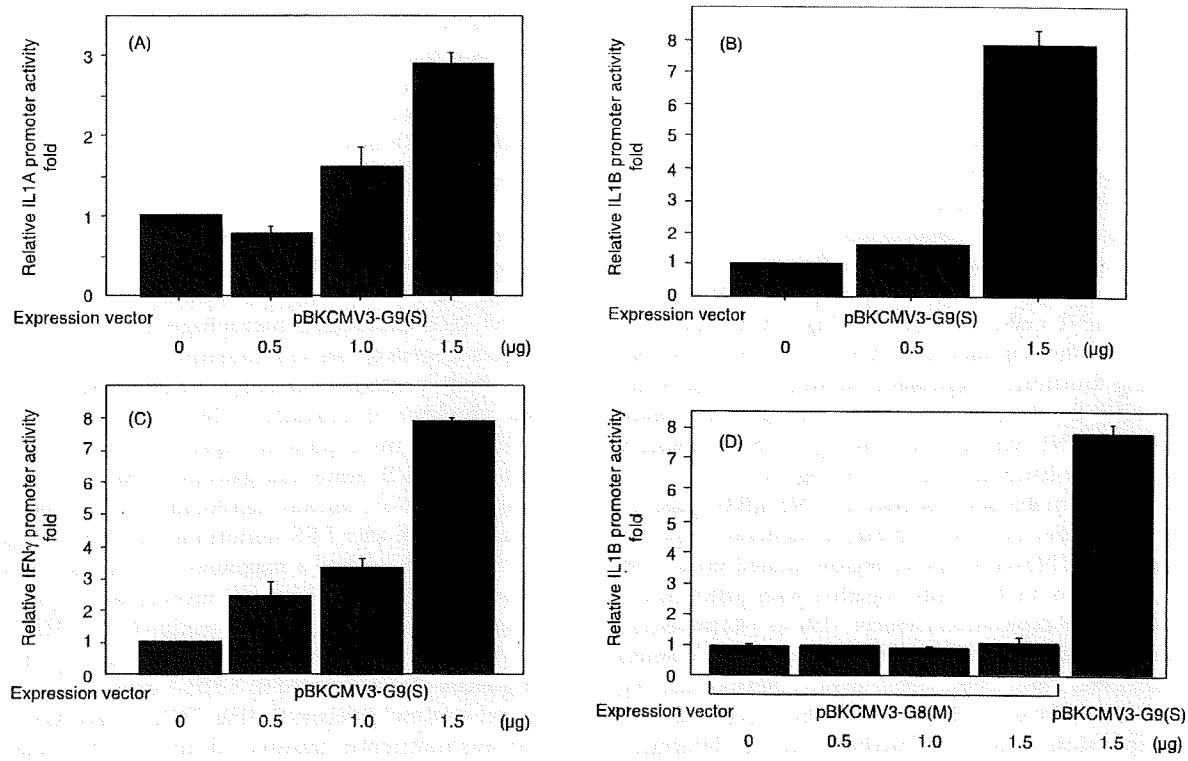

Figure 3 Galectin-9 activates cytokine gene promoters in THP-1 cells. pBKCMV3-G9(S) (0.5–1.5 μg) was transiently transfected together with 1.0 μg of cytokine promoter reporters [(A) IL1A, (B) IL1B, (C) IFNγ] into THP-1 cells. The total amount of transfected DNA was kept constant (3.0 μg) by adding control vector. Data are mean ± SD of triplicate samples. Luciferase assays were carried out as described in Experimental Procedures. Data were normalized by internal control Renilla luciferase activity.

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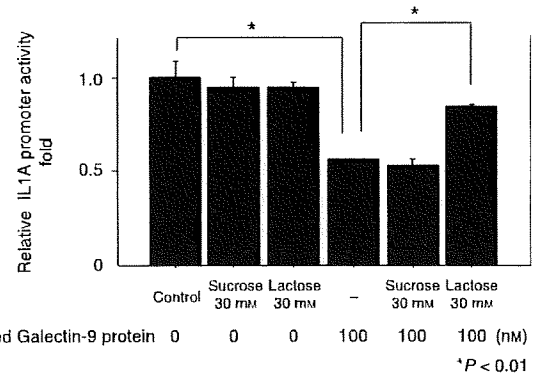

Figure 4 Exogenous galectin-9 protein inhibits *IL1A* promoter activity in THP-1 cells. pGL3IL1α (1.0 μg) was transfected into THP-1 cells. The cells were incubated with or without 30 mM lactose or 30 mM sucrose for 24 h, followed by stimulation with 100 nM galectin-9 protein (hG9NC (null)) for 24 h. Cell were collected and assayed for *IL1A* promoter activity. The total amount of transfected DNA was kept constant (3 μg) by the addition of control vector. Data are mean ± SD of triplicate samples. Luciferase assays were carried out as described in Experimental Procedures. Data were normalized by internal control Renilla luciferase activity.

transcription factors such as NF-κB, NF-IL6 and AP-1 to their target sites within the promoter lesions. NF-IL6 transactivates *IL1B* gene promoter through its binding to two different sites, −91 to −83 and −41 to −33 (Natsuka *et al.* 1992; Kominato *et al.* 1995). Based on our finding that intracellular galectin-9 induces inflammatory cytokines in monocytes, we co-transfected pG2mfNFκB, pG3mfNF6 × 3 and pAP1(1)-Luc, along with pBKCMV3-G9(S), into THP-1 cells. As shown in Fig. 5A,B, galectin-9 enhanced the activities of both AP-1 and NF-IL6, suggesting functional cooperation between galectin-9 and the bZIP family members. In contrast, galectin-9 failed to induce NF-κB activity, although LPS significantly induced the latter in THP-1 cells (Fig. 5C).

To confirm the lack of activation of NF-κB by galectin-9, we transfected pGL3TNFα, a luciferase reporter harboring TNF-α promoter region located from −199 to +82, into THP-1 cells, together with pBKCMV3-G9(S). TNF-α promoter element −199 to +82 possesses a binding site for NF-κB, but not NF-IL6 or AP-1 (Natsuka *et al.* 1992; Geist *et al.* 1997). pGL3TNFα containing the NF-κB site was activated by LPS, but not by galectin-9 expression (Fig. 5D). Furthermore, to determine whether −91 to −83 NF-IL6 site within the IL1B promoter plays a pivotal role in the transactivation of the IL1B promoter by galectin-9, a mutated luciferase reporter, pGL3HTmNF-IL6 was used. HTmNF-IL6 was identical to the wild-type HT, but contained nucleotide substitutions within the −91 to −83 NF-IL6 binding site. As a result, mutation of the −91 to −83 NF-IL6 binding site completely prevented activation by galectin-9 (Fig. 5E).

Physical association of intracellular galectin-9 with NF-IL6

The finding that galectin-9 induced NF-IL6 activity in THP-1 cells led us to examine the physical association of galectin-9 with NF-IL6. In these experiments, pcNFIL6 and/or pBKCMV3-G9(S) were transfected into HEK293T cells and 24 h after transfection, the cell lysates were immunoprecipitated with anti-galectin-9 Ab followed by WB using anti-NF-IL6 Ab. No expression of galectin-9 was detected in HEK293T transfected with a mock vector (Fig. 6A). Anti-galectin-9 Ab immunoprecipitation contained NF-IL6, only when both of pcNFIL6 and pBKCMV-G9(S) were transfected into HEK293T cells, suggesting the association of NF-IL6 with galectin-9 in the absence of DNA (Fig. 6A). In addition, pBKCMV3-G9(S) transfection into HEK293T cells also induced NF-IL6 activity (data not shown). Moreover, our IP/WB data using anti-gelectin-9 Ab showed a single galectin-9 protein band only when pBKCMV3-G9(S) was transfected into HEK293T cells, indicating the specificity of anti-galectin-9 Ab used in the present study to galectin-9 protein (Fig. 6B).

Galectin-9 is an LPS-responsive factor

LPS functions as a mediator of various inflammatory responses to activate cytokine genes such as IL-1α, IL-1β and TNF-α in monocytes/macrophages and is involved in the pathogenesis of various inflammatory diseases. In the present study, confocal microscopy identified galectin-9 in the cytoplasm of untreated THP-1 monocytic cells (Fig. 7A). Galectin-9 protein expression was further confirmed in WB using anti-galectin-9 Ab (data not shown). In addition, nuclear translocation of galectin-9 was observed 1 h after LPS stimulation of THP-1 cells (Fig. 7A,B), and reached a maximum at 6 h after LPS treatment (Fig. 7A,B). Next, we investigated physical interaction of NF-IL6 with galectin-9 in THP-1 cells. As no co-immunoprecipitation of NF-IL6 with galectin-9 was detected in THP-1 cells which did not over-express galectin-9 (Fig. 7C), pBKCMV3-G9(S) was transiently transfected into THP-1 cells. In THP-1 cells carrying pBKCMV3-G9(S), co-immunoprecipitaion of NF-IL6 with galectin-9 was observed. Treatment of THP-1 cells with LPS further enhanced co-immunoprecipitaion of NF-IL6 with

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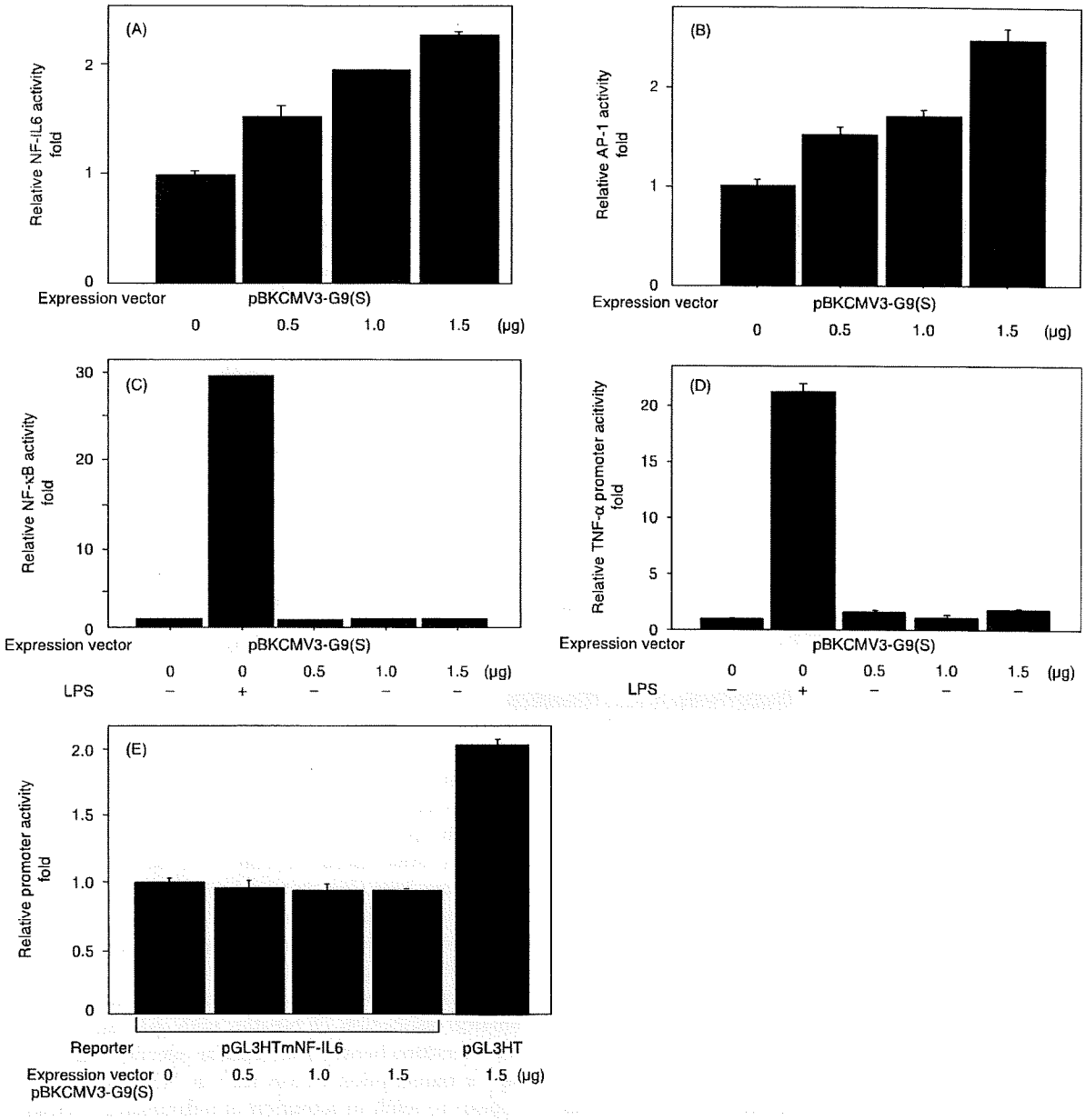

Figure 5 Effects of galectin-9 on various transcriptional factors in THP-1 cells. pBKCMV3-G9(S) (0.5–1.5 μg) was transiently transfected into THP-1 cells together with 1.0 μg of pG3mfNF6 × 3 (A), pAP1(1)-Luc (B), pG2mfNFκB (C), pGL3TNFα (D) and PGL3HTmNF-IL6 (E). The total amount of transfected DNA was kept constant (3.0 μg) by the addition of control vector. Data are mean ± SD of triplicate samples. Luciferase assays were carried out as described in Experimental Procedures. Data were normalized by internal control Renilla luciferase activity. As a positive control, THP-1 cells were treated with LPS (C and D).

galectin-9 (Fig. 7C). In addition, when THP-1 cells carrying pBKCMV3-G9(S) were treated with LPS, galectin-9 synergized with LPS to activate NF-IL6 in THP-1 cells (Fig. 7D). These data clearly demonstrated cooperativity of galectin-9 with NF-IL6.

Discussion

Galectin-9 was first described in Hodgkin's lymphoma as a tumor antigen. Galectin-9 exhibits various biological functions such as cell aggregation, chemoattraction and

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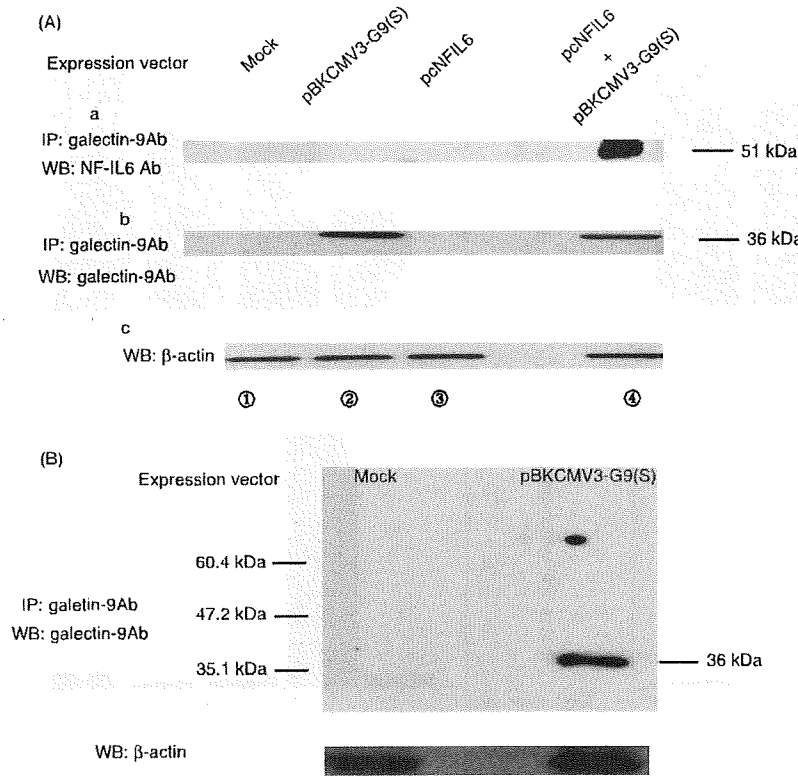

Figure 6 Galectin-9 physically interacts with NF-IL6 in HEK293T cells. (A) Cells lysates prepared from HEK293T cells transfected with pcNFIL6 and/or pBKCMV3-G9(S) were immunoprecipitated with anti-galectin-9 Ab and then blotted with anti-NF-IL6 Ab. Lane 1; Mock transfection, lane 2; pBKCMV3-G9(S) transfection, lane 3; pcNFIL6 transfection, lane 4; co-transfection of both pBKCMV3-G9(S) and pcNFIL6. IP-WB assay were carried out as described in Experimental Procedures section. Representative results of three experiments with similar findings. (B) Cells lysates prepared from HEK293T cells transfected with pBKCMV3-G9(S) or a mock vector. IP-WB assay were carried out as described in Experimental Procedures. Representative results of three experiments with similar findings.

apoptosis. A wide variety of cells including monocytes/macrophages express galectin-9. Galectin-9 expression has been reported to be down-regulated during monocytic differentiation of HL60 cells (Kashio *et al.* 2003). In contrast, a recent study demonstrated that LPS enhances expression of galectin-9 protein and mRNA in the periodontal ligament cells (Kasamatsu *et al.* 2005). In our study, the results of confocal microscopy indicated evident expression of galectin-9 protein in untreated THP-1 monocytes. Moreover, it is interesting to note that stimulation of THP-1 monocytic cells with LPS induced nuclear translocation of galectin-9 within 1 h, that is, galectin-9 is an LPS-responsive protein in monocytes (Fig. 7). We also demonstrated that intracellular galectin-9 in THP-1 monocytes induced the expression of both IL-1α and IL-1β mRNAs (Fig. 1A,B) and transactivation of inflammatory cytokine gene promoters, such as *IL1A*, *IL1B* and *IFNγ* (Fig. 3A–C). In contrast, extracellular gelectin-9 failed to activate the *IL1A* promoter in the same cells. Interestingly, our transient transfection data further showed that intracellular galectin-9 induced the activation of transcription factors such as NF-IL6 and AP-1 (Fig. 5A,B). No activation of NF-κB by intracellular galectin-9 was observed in THP-1 cells (Fig. 5C). These results strongly suggest that induction of inflammatory cytokine gene promoters is mediated through activation of leucin zipper transcription factors such as NF-IL6 and AP-1 by intracellular galectin-9. In fact, NF-IL6 and AP-1 have been demonstrated to be involved in trans-activation of the inflammatory cytokine genes (Akira & Kishimoto 1992). Furthermore, our IP/WB using HEK293T cells and THP-1 cells showed co-immuno-precipitation of NF-IL6 with galectin-9, indicating direct physical association of NF-IL6 with galectin-9. Thus, the interaction between intracellular galectin-9 and leucin zipper transcription factors such as NF-IL6 and AP-1 appears to result in activation of inflammatory cytokine genes. Moreover, since six different members of C/EBPs have been identified, our data may suggest the possibility that galectin-9 cooperates with the other C/EBP family proteins. Considered together, the above results emphasize the role of nuclear galectin-9 in inflammatory gene-regulation.

NF-IL6 (C/EBP β), a member of the basic leucine zipper (bZIP) family of transcription factors was initially identified as a nuclear factor that binds to the IL-1 responsive elements (Akira *et al.* 1990). NF-IL6 also plays a role in regulation of the genes encoding several

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acute-phase protein genes, albumin, c-fos, and adipocyte specific proteins (Poli *et al.* 1990; Isshiki *et al.* 1991). Furthermore, various genes involved in inflammatory and immune responses, including IL-8, granulocyte/colony-stimulating factor, IL-1 and immunoglobulin genes, have been demonstrated to be activated by NF-IL6 (Akira & Kishimoto 1992). NF-IL6 is present ubiquitously in cells in an inactive protein form that support transcription following posttranslational modification and/or physical association with other transcription factors. The role of NF-IL6 as an LPS-responsive transcription factor in a pre-existing inactive form in unstimulated monocytes/macrophages has been established (Shirakawa *et al.* 1993).

In addition, NF-IL6 is heterodimerized with members of the C/EBP family and the AP-1 family through their bZIP regions and is also associated with several transcription factors outside the bZIP family members such as NF-κB (Stein *et al.* 1993), Tax (Tsukada *et al.* 1997), PU.1 (Yang *et al.* 2000), heat shock factor 1 (Xie *et al.* 2002), calcium channel blocker (CCB) (Eickelberg *et al.* 1999), chicken ovalbumin upstream promoter transcriptional factor (COUP-TF) (Schwartz *et al.* 2000) and glucocorticoid receptor (GR) (Nishio *et al.* 1993). Our previous study reported that NF-IL6 vigorously activates IL-1β core promoter via protein-tethered transactivation mediated by PU.1 (Yang *et al.* 2000). In the present study, we further demonstrated that intracellular galectin-9 transactivated inflammatory cytokine genes through physical association with NF-IL6 in monocytes. In contrast, extracellular galectin-9 has been reported to induce apoptosis not only of T cells but also of B cells (BALL-1), monocytes (THP-1), and myelocytes (HL-60), when these cells were treated with 1 μM galectin-9 for 24 h (Kashio *et al.* 2003), indicating that extracellular galectin-9 can function as a proapoptotic factor for various immune cells. Our transient transfection studies also showed partial inhibition of *IL1A* gene promoter activity in THP-1 cells incubated with exogenous galectin-9 (Fig. 4). These results demonstrate distinct functions for extracellular and intracellular gelectin-9.

There are only a few reports regarding intracellular functions of galectins. Nuclear export of phosphorylated galectin-3 prevents apoptosis through the c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways (Takenaka *et al.* 2004). Galectin-3 is involved in RNA processing and cell cycle-regulation through activation of transcription factors when translocated to the nucleus. The nuclear localization of galectin-3, a pre-mRNA splicing factor is associated with proliferation of normal cells. Galectin-3 located in nuclei of papillary cancer cells up-regulates transcriptional activity of a thyroid-specific transcription factor TTF-1 via interaction between with TTF-1 homeodomain (Paron *et al.* 2003). Cyclin D1 promoter is also enhanced through enhancement/stabilization of CRE-associated complex formation by gelectin-3 (Lin *et al.* 2002). In contrast, galectin-4 is considered to be involved in p27-mediated activation of the myelin basic protein promoter via its physical interaction with p27 (Wei *et al.* 2007).

Monocytes are instrumental in mediating immediate immune and inflammatory responses. Various important inflammatory and immuno-regulatory cytokines are expressed by activated monocytes/macrophages. These cells can respond almost instantly to a variety of stimuli including LPS, IL-1 and other cytokines, and undergo a rapid transformation from resting monocytes to activated ones. The inflammatory cytokine genes in resting monocytes/macrophages are also silent, but rapidly transcribed in competent cells on stimulation. Production of inflammatory cytokines is tightly regulated in monocytes/macrophages. (Auron & Webb 1994). In this regard, it is noteworthy that galectin-9 is a LPS-responsive factor, which can form a complex with NF-IL6 to transactivate inflammatory cytokine gene promoters in monocytes/macrophages. In conclusion, the present study demonstrated a novel function for gelectin-9; transactivation of inflammatory cytokine genes in monocytes through physical interaction with leucin zipper transcription factors such as NF-IL6.

Experimental procedures

Cell cultures

Human THP-1 monocytic cell line (JCRB0112) was purchased from Health Science Research Resource Bank (Osaka, Japan), and human endothelial kidney 293T cells (HEK293T cells) were used in the present study. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 0.5% of penicillin and streptomycin in a humidified incubator under 5% CO_2 at 37 °C. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS. Cells were split at one-third of dilution every 3 or 4 days to avoid over-crowding and were further split at 1 : 2 on the day before transfection.

Plasmids

Human IL-1β gene (*IL1B*) promoter region (HT sequence) located between positions −131 to +12 (pGL3HT), IFN-γ promoter sequence from −572 to +75 (pGL3IFNG), human IL-1α gene (*IL1A*) promoter fragment from −1437 to +725 (pGL3IL1α), TNFα promoter region located from −199 to +82 (pGL3TNFα) were inserted into pGL3-basic luciferase reporter (Promega,

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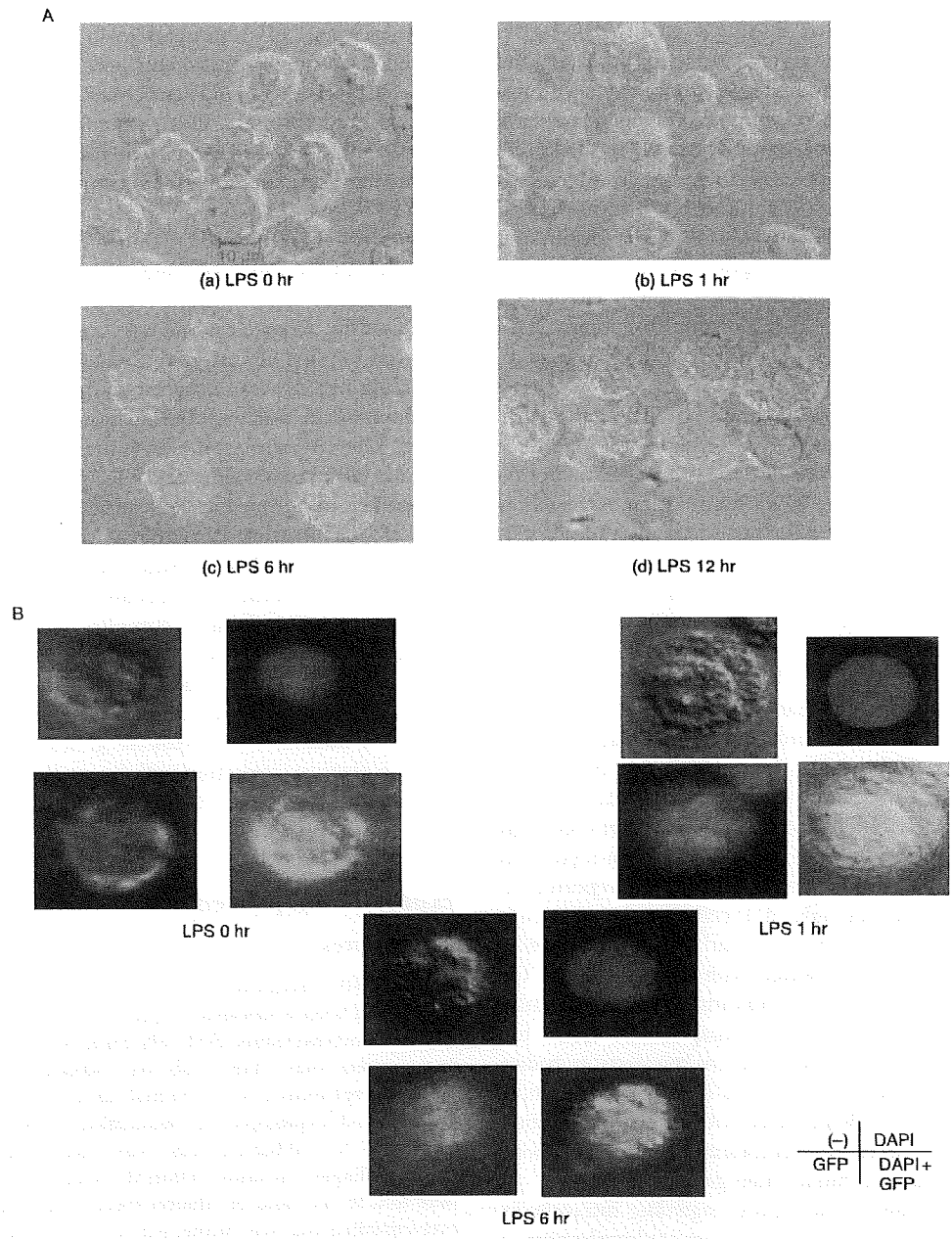

Figure 7 Galectin-9 is an LPS-responsive factor, which is translocated into the nucleus and is physically associated with NF-IL6 following treatment of THP-1 cells with LPS. (A) THP-1 monocytic cells were stimulated with LPS (1 μg/mL) or left untreated. Confocal microscopy analysis for galectin-9 was carried out as described in Experimental Procedures. Magnification, 63× and 1.4 oil DIC for all panels (scale bar = 10 μm). (B) Nuclear translocation of galectin-9 was observed after stimulation of LPS by fluorescence microscopy with DAPI stain (blue). (C) THP-1 cells were transfected with pBKCMV3-G9(S) (1.5 μg). 24 h after transfection, cells were treated with LPS for 6 h, or left untreated. IP-WB assay were carried out as described in Experimental Procedures. Representative results of three experiments with similar findings. (D) pBKCMV3-G9(S) (1.0 μg) was transiently transfected together with 1.0 μg of pG3mfNF6 × 3 into THP-1 cells. Cells were stimulated with LPS (0.5 μg/mL) or left untreated. The total amounts of transfected DNA was kept constant (3.0 μg) by adding control vector. Data are mean ± SD of triplicate samples. Luciferase assays were carried out as described in Experimental Procedures section. Data were normalized by internal control Renilla luciferase activity.

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Madison, WI), respectively (Mizobe *et al.* 2007). Mutations of the −91 to −83 upstream NF-IL6 binding site within the *IL1B* promoter were the same as those reported previously (Kominato *et al.* 1995). HTmNF-IL6 (the core sequence 5′-gTGTtAAgT-3′) was identical to the wild-type HT, but contained nucleotide substitutions within the −91 to −83 NF-IL6 binding site. pG2mfNFκB; NF-κB luciferase reporter and pG3mfNF6 × 3; NF-IL6 luciferase reporter were described previously (Mizobe *et al.* 2007). pAP1(1)-Luc designed for monitoring induction of activator protein (AP)-1 and stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) signal transduction pathway was purchased from Panomics (Redwood City, CA).

The expression vectors for galectin-9(S), galectin-9(M) and galectin-9(L) were pBKCMV3-G9(S), pBKCMV3-G9(M) and pBKCMV3-G9(L), respectively. Galectin-9 is a tandem-repeat type and more susceptible to proteolysis than other galectins due to the presence of a relatively long linker peptide. We found protease-resistant galectin-9 by modification of its linker peptide, hG9NC (null). The NF-IL6 (C/EBP β) expression vector pcNFIL6 was described previously (Tsukada *et al.* 1997). pBKCMV3-G8(M) was a galectin-8 expression vector.

Confocal and fluorescence microscopy

THP-1 cells (1×10^5) were collected and treated with 4% formaldehyde (Sigma Chemical Co., St. Louis, MO) in FACS medium for 15 min. and then with 0.1% saponin (Sigma) in FACS medium. The cells were incubated with a specific antibody (Ab) against galectin-9 (GalPharma, Kagawa, Japan) for 30 min at 4 °C. Subsequently, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG Ab at saturating concentrations in FACS medium. We performed confocal analysis of galectin-9 using an inverted laser scan microscope (model LSM5-pascal, Carl Zeiss Microscope System, Germany). For detection of DNA/nuclei using 4′,6-diamidino-2-phenylindole (DAPI), section were incubated for 2 min in a 300-nM solution of DAPI dilactate in PBS. Cells were overlaid and observed by fluorescence microscopy (Axiovert 135 inverted microscope; Zeiss, oberkochen, Germany) for enhanced green fluorescent protein (eGFP)-galectin-9 (green) and DAPI nuclear staining (blue). Green and blue channel images were merged using Axio-Vision software (Zeiss).

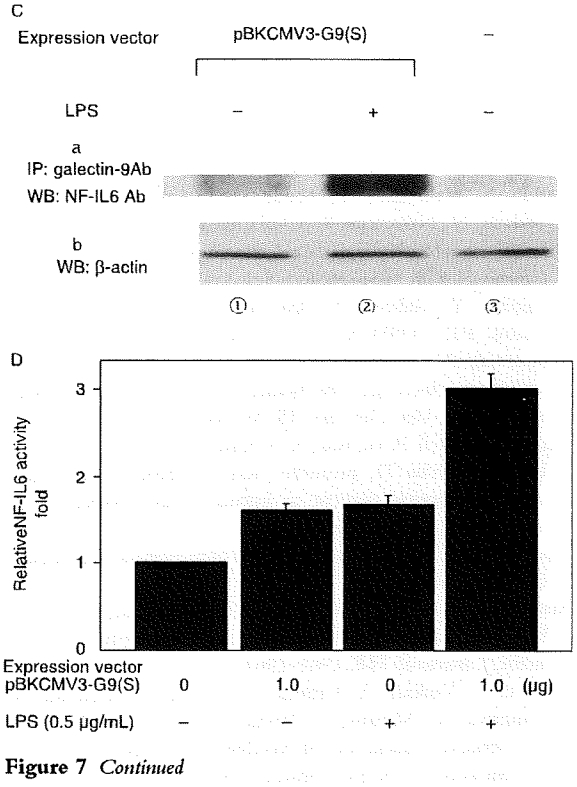

Figure 7 *Continued*

Transfections and luciferase assays

THP-1 cells were transfected by the DEAE-dextran methods as described previously (Shirakawa *et al.* 1993; Tsukada *et al.* 1997). This technique was used because, unlike electroporation, it did not induce endogenous *IL1A* and *IL1B*. Cells (2×10^6 cells per plate) were transfected with 3 μg of plasmids. Transfection of plasmids into HEK-293T cells was carried out using a transfection kit; Transfast (Promega) according to the protocol recommended by the manufacturer. At 24 h after transfection, cells were lysed with Passive Lysis Buffer (Promega). The cell lysates were used for a dual-luciferase reporter assay system (Promega). Samples were normalized to Renilla luciferase activity as an internal control for transfection efficiency.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of THP-1 cells were extracted by Isogen RNA extraction kit (Nippon Gene, Tokyo). Total RNA (0.5 μg) was used along with a reverse transcriptase RNA PCR kit; Access RT-PCR System (Promega) according to the instructions provided by the supplier. An aliquot of the PCR mixture was subjected to electrophoresis in 3% agarose gel. The primers used were human IL-1α sense, 5′-GTCTCTGAATCAGAAATCCTTCTATC-3′; human IL-1α anti-sense, 5′-CATGTCAAATTTCACTGCTTCATCC-3′; human IL-1β sense, 5′-CAGAGAGTCCTGTGCTGAAT-3′; human IL-1β anti-sense, 5′-GTAGGAGAGGTCAGAGAGGC-3′ (Herbein *et al.* 1994), β-actin sense, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′; and β-actin anti-sense, 5′-CCTAGAAGCATTTGCGGTGCAAGATG-3′.

Protein extraction, immunoprecipitation (IP) and Western blotting (WB)

Cells pellet was lysed with lysis buffer (in mM, 150 NaCl, 50 Tris–HCl, 2 sodium orthovanadate, and 5 sodium pyrophosphate) with protease inhibitor mixture (complete mini) and 1% Triton X-100. The same amount of 2 × SDS (sodium dodecylsulfate) buffer

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(125 mM Tris–HCl, 4% SDS, 20% glycerol, and 0.02% bromophenol blue) with 2-mercaptoethanol was added to each sample, then boiled for 3 min. Cells lysates were incubated with anti-galectin-9 Ab and 50 μL of Protein G Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 0.5 h on ice. Anti-NF-IL6 Ab (Santa Cruz Biotechnology Inc.) was used for WB. IP and WB were carried out as described previously (Mizobe *et al.* 2007).

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student's *t*-test. A P value < 0.05 denoted the presence of a statistically significant difference.

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Postmarketing Surveillance of the Safety and Effectiveness of Etanercept in Japan

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ABSTRACT. ***Objective.*** Postmarketing surveillance (PMS) was conducted evaluating safety and effectiveness of etanercept (ETN; Enbrel®) in Japan, following all patients with rheumatoid arthritis (RA) during the conditional approval period of ETN.

Methods. Registration of patients from 1,334 medical sites was conducted between March 2005 and April 2007. Patients were followed for 24 weeks; data regarding patients' background, safety, and effectiveness was recorded centrally. Adverse events (AE) and adverse drug reactions (ADR) were coded using the Medical Dictionary for Regulatory Activities. Effectiveness was measured using the Disease Activity Score 28 (DAS28).

Results. Of 14,369 patients registered, data collection and evaluation for 7,091 patients by March 2006 is reported. At least 1 AE was observed for 2,173 patients (30.6%); 60% of AE occurred within 8 weeks of starting ETN. Most frequent AE were injection site reaction (n = 377, 5.3%) and rash (n = 228, 3.2%). Serious AE occurred in 403 patients (5.7%); most frequent were pneumonia (n = 59, 0.8%) and interstitial lung disease (n = 42, 0.6%). Pneumonia was the most common specifically important ADR (n = 102, 1.4%). Mean baseline DAS28 was 6.0, which reduced to 4.4 within 4 weeks, and to 3.9 within 24 weeks. The proportion of patients having good or moderate EULAR response measured by DAS28 was 84.1% at Week 24. Effectiveness rates were more favorable in patients concomitantly using methotrexate. Good or moderate EULAR response rate among patients switched from infliximab was 84.9%.

Conclusion. This extensive observational trial, including all patients with RA in Japan taking ETN, found ETN to be both effective and well tolerated by Japanese patients with RA. Trial registration: clinicaltrials.gov identifier NCT00503503. (First Release April 1 2009; J Rheumatol 2009;36:898–906; doi:10.3899/jrheum.080791)

Key Indexing Terms:
RHEUMATOID ARTHRITIS ETANERCEPT POSTMARKETING SURVEILLANCE
JAPAN EFFICACY SAFETY

Phase III clinical trials are intended to evaluate efficacy and safety of new drugs. However, even large well designed Phase III clinical studies may fail to reflect the safety and effectiveness of new drugs in the real world. The goal of postmarketing surveillance (PMS) is to monitor ongoing safety and effectiveness of marketed drugs by identifying adverse events (AE), adverse drug reactions (ADR), and effectiveness. Risks and benefits associated with taking a particular drug are reassessed based on information learned after the drug is marketed, and ways to appropriately balance the risks and benefits are then recommended. In the US, PMS is overseen by the Food and Drug Administration, operating through a system called Medwatch[1], while in Canada, a division of Health Canada, the Marketed Health Products Directorate, coordinates it[2]. In Japan, the Pharmaceutical and Medical Device Agency (PMDA) is the corresponding regulatory body.

Etanercept (ETN; Enbrel®) is a biologic agent used to treat patients with rheumatoid arthritis (RA) who have inadequately responded to at least one other disease modifying antirheumatic drug (DMARD), often including methotrexate (MTX)[3,4]. In the ERA[5] (Early Rheumatoid Arthritis) and COMET[6] (Combination of Methotrexate and Etanercept in

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Active Early Rheumatoid Arthritis) trials MTX- and DMARD-naive patients were treated. ETN is a fully human soluble receptor Fc fusion protein that binds to tumor necrosis factor (TNF) and blocks its interaction with cell-surface TNF receptors. In Japan, the use of ETN for RA was conditionally approved in March 2005. ETN could be used only when the medical institution fulfilled the institution selection criteria defined by the PMDA. All physicians prescribing ETN and patients using ETN were registered in the All Cases Surveillance program. A further condition of approval was the collection of AE of special interest, specifically tuberculosis (TB), opportunistic infection, interstitial lung disease, malignancy, demyelinating diseases, and congestive heart failure.

This PMS registered all patients treated with ETN in Japan. Information about onset, frequency, severity, and other details of AE, ADR, and other factors considered related to safety and effectiveness was collected. The primary objective of surveillance was to evaluate the real-world safety and effectiveness of ETN for all patients receiving it in Japan after its introduction into the market. The PMS study for ETN began in March 2005 and registered more than 14,000 patients in 2 years. This report presents safety and effectiveness results from the 7,091 patients registered between March 2005 and March 2006 who completed 24 weeks of ETN treatment and observation.

MATERIALS AND METHODS

There were 1,334 sites participating in the study (trial registration identifier NCT00503503); every RA patient treated with ETN in Japan between March 2005 and March 2006 was tracked for the first 24 weeks of treatment. Patient eligibility for treatment with ETN was based on the Japan College of Rheumatology treatment guidelines[7]. Patients were those with RA who had not been successfully treated with other conventional DMARD (MTX, salazosulfapyridine, bucillamine) and who had ≥ 6 tender joints, ≥ 6 swollen joints, erythrocyte sedimentation rate (ESR) ≥ 28 mm/h, or C-reactive protein (CRP) ≥ 2.0 mg/dl. They also met the following criteria: a low risk for opportunistic infections as defined by a white blood cell count ≥ 4,000/mm^3, peripheral blood lymphocytes ≥ 1,000/mm^3, and negative serum ß-D-glucan. Mandatory chest radiographs and tuberculin tests were also obtained prior to the initiation of ETN. ETN (10 mg or 25 mg) was administered by a single subcutaneous injection twice a week. Patients had training for self-injection over a 1-month period and were subsequently allowed to proceed with self-injection after learning the skills.

Data recording. The PMS protocol was reviewed and approved by the Ministry of Health, Labor and Welfare. Registration was conducted centrally at the time patients started ETN, and data collection was performed through an electronic data capture system, the Internet, or hardcopy case report forms. Medical representatives from Wyeth and Takeda Pharmaceutical Company visited sites periodically to collect additional data as required. Information on age, sex, comorbidity, Steinbrocker radiographic stage[8], Steinbrocker functional class, duration of RA, history of smoking, previous and concomitant use of steroids, and concomitant use of DMARD including MTX was collected. ESR and CRP levels were also measured and recorded.

Safety evaluation. All patients were assessed for safety every 2 weeks. Safety evaluation included all events that occurred within 24 weeks of the first dose of ETN and within 30 days of the last dose of ETN. Particular attention was paid to monitoring the occurrence of infections, especially TB. Safety data were coded with preferred terms from the *Medical Dictionary for Regulatory Activities* (MedDRA)[9]. Definition of AE (untoward medical events not necessarily causally related to the treatment) and ADR (noxious and unintended responses deemed to be related to the treatment) were based on the ICH Harmonised Tripartite Guideline[10]. Safety information obtained through PMS was evaluated independently by the Japan College of Rheumatology PMS Committee.

Effectiveness evaluation. Effectiveness of treatment was measured by European League Against Rheumatism (EULAR) response criteria[11] and Disease Activity Score (DAS28)[12]. The DAS28 is a combined index of 28-joint evaluation, ESR, and pain on visual analog scale. DAS28 was divided into 4 categories: remission (≤ 2.6), low disease activity (> 2.6 and ≤ 3.2), moderate disease activity (> 3.2 and ≤ 5.1), and high disease activity (> 5.1). Good response was defined as improvement > 1.2 on the DAS28 from baseline, and a DAS28 score obtained during followup of ≤ 3.2. Nonresponders were patients with improvement of ≤ 0.6 or patients with improvement between 0.6 and 1.2 and a DAS28 during followup of > 5.1. The remaining patients were classified as moderate responders, defined as an improvement on the DAS28 from baseline of between 0.6 and 1.2, and a DAS28 obtained during followup of ≤ 5.1. Treatment was defined as effective for cases with a moderate or good response. Morning stiffness was measured in addition to DAS28.

Data analysis. The cumulative occurrence rates of AE and ADR were compiled to generate a Kaplan-Meier plot. Missing data values were processed using the last observation carried forward method, except baseline values, which were not carried forward. For example, if the value at Week 4 was missing, the value at Week 8 was present, and all values at Week 12 and thereafter were missing, then the value at Week 8 would be substituted for all the values at Week 12 and thereafter (but the value at Week 4 would remain as missing). Paired sample t tests were used to compare differences between baseline and Weeks 4 and 24, chi-square tests to compare the differences in EULAR response rates, and Cochran-Armitage tests to evaluate trends in response rates.

RESULTS

Results of the interim analysis of the PMS are presented below. The authors plan to present the results of the final analysis of all 14,369 registered patients when the study is complete.

Patient characteristics. A total of 7,091 patients treated with ETN completed 24 weeks of treatment and observation during the study period (Table 1). A majority (81%, n = 5,746) were female. Most patients (79%, n = 5,600) were over 50 years of age, and mean patient weight was 53.3 kg (n = 4,455). Of total cases, 7,066 cases (99.6% correction rate) were treated for RA — the indications that were not RA (25 cases in total) were "malignant RA" (RA with rheumatoid vasculitis, 11 cases), juvenile idiopathic arthritis (5 cases), adult-onset Still's disease (5 cases), pustulotic arthroosteitis (1 case), multicentric reticulohistiocytosis (1 case), Takayasu arteritis (1 case), and sarcoidosis (1 case). Most patients (71%, n = 5,037) used one or more DMARD concomitantly with ETN, and 52.7% of patients (n = 3,736) received MTX. Most patients (75%, n = 5,317) received concomitant corticosteroids. A medical history of TB was present in 453 patients (6.5%), of whom 98% (n = 444) received chemoprophylaxis for TB (isoniazid; INH). Nearly every patient (98.5%, n = 6,988) received a chest radiograph, and 93.8% (n = 6,649) received tuberculin tests.

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Table 1. Characteristics of patients participating in postmarketing surveillance of etanercept (ETN) in Japan.

Characteristic	ETN Cases, n (%)
Male/female	1345/5746 (19.0/81.0)
Age, yrs, 15–19, 20–29, 30–39, 40–49, 50–59, 60–69, ≥ 70	32 (0.5), 190 (2.7), 468 (6.6), 801 (11.3), 1983 (28.0), 2212 (31.2), 1405 (19.8)
Mean weight, kg (range)	53.3 (27–130)
Steinbrocker* stage: I, II, III, IV	368 (5.2), 1551 (21.9), 2598 (36.7), 2567 (36.2)
Steinbrocker* function: I, II, III, IV	498 (7.0), 3984 (56.2) 2343 (33.1), 259 (3.7)
History of tuberculosis: yes	453†† (6.5)
Comorbidities: yes	4128 (58.2)
History of smoking: yes	1030 (16.5)
History of steroid use: yes	6066 (88.1)
No. patients receiving	
Chest radiographs	6988 (98.5)
Tuberculin tests	6649 (93.8)
Dose of ETN† administered to patients, no. of patients (%)	
50 mg	5866 (82.7)
20 mg	14 (0.2)
20–50 mg	1077 (15.2)
Concomitant use of DMARD: yes	5037 (71.0)
No. concomitant DMARD: 1/2/3/4	3758/1117/144/18 (53.0/15.8/2.0/0.3)
Dose of MTX	
No MTX	3355 (47.3)
< 6 mg	1076 (15.3)
≥ 6 mg ≤ 8 mg	1273 (18.0)
> 8 mg	1387 (19.6)
Concomitant use of steroid: yes	5317 (75.0)
History of infliximab use: yes	808 (11.4)

DMARD: disease modifying antirheumatic drug; MTX: methotrexate. * A system to define RA, classify the stages of RA progression, criteria for therapeutic response of RA disease activity, and classification of functional impairment[8]. † Dose and administration in Japanese labeling is 10 to 25 mg × 2 per week. †† 444 (98%) received chemoprophylaxis (isoniazid).

These results suggest that the PMS program successfully encouraged physicians to conduct the appropriate screening and chemoprophylaxis for TB before starting ETN. The ETN dose regimen received most often by patients (82.7%, n = 5,866) was 50 mg per week (25 mg × 2 doses); 808 patients (11.4%) had previous treatment with infliximab.

Safety. All 7,091 cases were included in safety analyses. Data collected for AE and ADR, coded using preferred terms from MedDRA, showed that the incidence of any AE or ADR was 35.3% (n = 2,506) and 30.6% (n = 2,173), respectively (Table 2A). Type and incidence of frequently observed AE or ADR are shown in Tables 2B and 2C. Injection site reaction was the most frequently observed AE (5.3%, n = 377), followed by rash (3.2%, n = 228) and pyrexia (2.4%, n = 169; Table 2B). The incidence of serious AE and serious ADR were 7.5% (n = 535) and 5.7% (n = 403), respectively (Table 2A). The most frequent serious AE was pneumonia (0.8%, n = 59) followed by interstitial lung disease (0.6%, n = 42; Table 2C). Specifically important ADR for patients with RA, especially while being treated with TNF inhibitors, are summarized in Table 3. Ten suspected TB cases were reported during the 24-week observation period. Of these 10 cases, 8 were confirmed as TB through at least 1 positive result by microscopic evaluation, polymerase chain reaction test, or culture. In 2 of the 8 confirmed cases, prophylactic INH had been received when ETN was started; retrospective evaluation suggested the existence of active TB at the onset of ETN treatment. Of the remaining 6 confirmed cases, prophylactic INH was not used prior to or during ETN treatment.

Fourteen cases of malignancy were reported during the observation period, which included 3 cases of lymphoma and 11 cases of malignant tumors. The average period between detection of malignancy and starting ETN was 82.1 days. Comments from the reporting physicians suggested that 8 out of 14 cases might have had preexisting malignancy when ETN was started. Two cases of congestive heart failure and one lupus-like syndrome were reported in this survey. There was no report of demyelinating disease. Among 7,091 patients, there were 29 deaths during the observation period of 24 weeks.

Continuation of etanercept. A total of 1,250 (17.6%) patients discontinued ETN during the observational period (Table 4). The percentage of patients continuing treatment declined from 96.0% after 4 weeks to 82.4% after 24 weeks (Figure 1). The primary reason for stopping treatment (Table

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Table 2A. Total number of adverse events and adverse drug reactions with etanercept.

	Etanercept All Cases (n = 7091)	
	Adverse Events, n (%)	Adverse Drug Reactions, n (%)
Total	2506 (35.3)	2173 (30.6)
Serious	535 (7.5)	403 (5.7)

Table 2B. Incidence of frequently observed adverse events with etanercept.

Adverse Event	n (%)
Injection site reaction	377 (5.3)
Rash	228 (3.2)
Pyrexia	169 (2.4)
Abnormal hepatic function	167 (2.4)
Nasopharyngitis	158 (2.2)
Pruritis	126 (1.8)
Upper respiratory tract infection	125 (1.8)
Pneumonia	88 (1.2)
Erythema	85 (1.2)
Herpes zoster	71 (1.0)

Table 2C. Incidence of frequently observed serious adverse events with etanercept.

Serious Adverse Event	n (%)
Pneumonia	59 (0.8)
Interstitial lung disease	42 (0.6)
Pyrexia	27 (0.4)
Sepsis	20 (0.3)
Herpes zoster	17 (0.2)
Pneumocystis jiroveci pneumonia	15 (0.2)
Urinary tract infection	13 (0.2)
Cellulitis	10 (0.1)
Bacterial arthritis	10 (0.1)
Femoral neck fracture	10 (0.1)

4) was AE (8.6%, n = 613), followed by lack of effectiveness (2.7%, n = 189) and by patients stopping their visits or moving to another hospital (2.4%, n = 169).

Kaplan-Meier analysis for cumulative rate of AE and ADR. A Kaplan-Meier analysis was performed (Figure 2) to evaluate the cumulative rate of AE and ADR. The occurrence rates of AE and ADR increased in a similar fashion until roughly Day 30. They continued to increase slowly until Day 180. Results also demonstrated that approximately 50% of ADR occurred within 8 weeks of initiating ETN.

Effectiveness. Of 7,091 cases, effectiveness was evaluated with 6,563 cases; 528 cases were excluded due to lack of assessable data (n = 478), too short treatment period (< 2 weeks; n = 25), etc. Several different measures including DAS28 were used to assess treatment effectiveness (Table 5). The evaluated number of each measure is shown in Table 5. The baseline average DAS28 score was 6.0; this reduced to 4.4 (27% improvement) within 4 weeks and further to 3.9

Table 3. Incidence of specifically important adverse drug reactions in patients participating in postmarketing surveillance of etanercept in Japan.

Adverse Drug Reactions	Etanercept n (%)
Total pneumonia*	102 (1.4)
Pneumocystis jiroveci pneumonia	16 (0.2)
Tuberculosis†	10 (0.1)
Pulmonary	8 (0.1)
Extrapulmonary††	2 (0.03)
Interstitial lung disease	44 (0.6)
Malignancy	14 (0.2)
Demyelinating disease	0 (0)
Congestive heart failure	2 (0.03)
Lupus-like syndrome	1 (0.01)

* Total pneumonia = pneumonia (n = 80), bacterial pneumonia (9), bronchopneumonia (6), chlamydia pneumonia (4), staphylococcal pneumonia (1), candida pneumonia (1), fungal pneumonia (1). One case developed both pneumonia and bronchopneumonia. † 2 indefinite cases. †† Tuberculous abscess (thigh) + gastrointestinal tuberculosis.

Table 4. Discontinuation of etanercept treatment.

Total no. patients who discontinued treatment (%)	1250 (17.6)
Period until discontinuing ETN, days	73.4 (± 48.5)
Reason for discontinuation, n (%)	
Adverse events	613 (8.6)
No effectiveness	189 (2.7)
Patient stopped visiting hospital/moved to another hospital	169 (2.4)
Other	142 (2.0)
Refused treatment for economic reasons	116 (1.6)
Achieved goal of treatment	21 (0.3)

ETN: etanercept.

(35% improvement) within 24 weeks of starting treatment ($p < 0.001$). The average duration of morning stiffness was 111.5 minutes at baseline, which declined to 44.3 minutes (60% improvement) within 4 weeks and to 31 minutes (72% improvement) within 24 weeks ($p < 0.001$). The number of tender joints (9.9; 4.5; 3.3) or swollen joints (9.1; 4.3; 3.0) and the visual analog scale score for pain (60.9 mm; 37.2 mm; 31.3 mm) also decreased significantly from baseline to Weeks 4 and 24, respectively. Baseline ESR value was significantly reduced from 61.1 to 39.3 mm/h (36% improvement) after 4 weeks and dropped to 37.1 mm/h (39% improvement) after 24 weeks ($p < 0.001$), while the baseline CRP value of 3.78 mg/dl dropped to 1.39 mg/dl (63% improvement) after 4 weeks and to 1.33 mg/dl (65% improvement) after 24 weeks ($p < 0.001$). Using EULAR response criteria, 1,780 out of 2,337 cases (76.2%) experienced a moderate or good response after Week 4; this improved to 3,268 out of 3,887 cases (84.1%) after 24 weeks (Figure 3). Positive response was greater among patients with concomitant use of MTX (Figure 4), and better effectiveness was observed with increasing MTX dose. For 411 patients previously treated with infliximab, 25.3%

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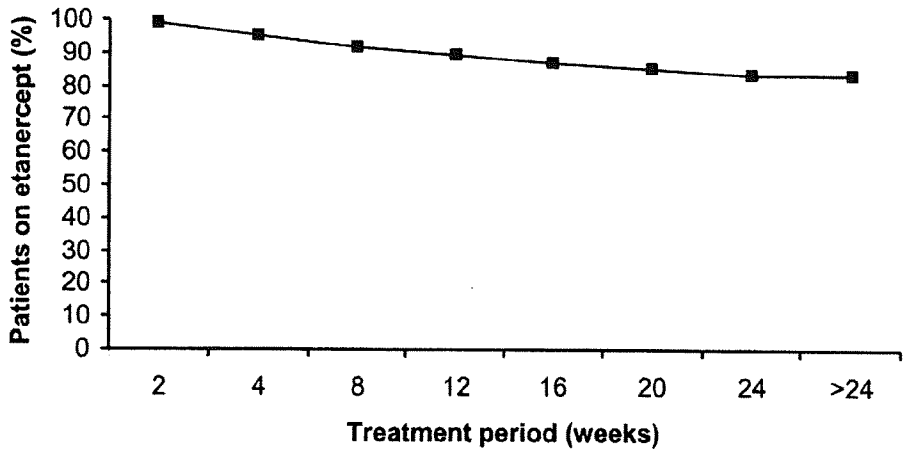

Figure 1. Continuation rate of patients taking etanercept.

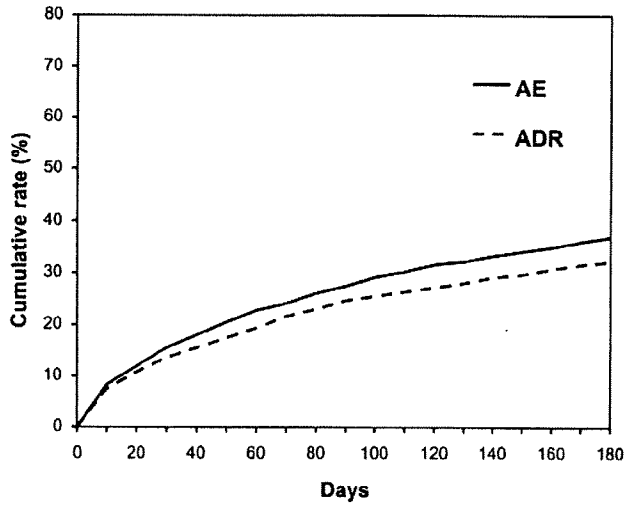

Figure 2. Kaplan-Meier analysis for cumulative rate of adverse events (AE) and adverse drug reactions (ADR) in patients taking etanercept.

(n = 104) achieved a good response while 59.6% (n = 245) achieved a moderate response (Figure 5).

DISCUSSION

This comprehensive PMS study followed all patients with RA treated with ETN in Japan. It was conducted in accord with the guidelines and instruction of the Japanese health authorities. Data monitoring and recording were achieved as originally planned, and all case report forms for the first 7,091 patients were collected successfully.

This study provides important information regarding real-world patient usage patterns of ETN. Given that several studies suggest that the outcome of DMARD treatment for RA may be better if it is initiated early in the course of the disease[13-15], studying the characteristics of patients registered in this PMS is useful. Results showed that 51% were age 60 years and older, 72% had advanced RA (Steinbrocker stage III and IV), and 58.2% had comorbidities. This emphasizes that positive results were also observed in the population despite starting treatment relatively late in the disease process. The overall safety and effectiveness profile in this PMS demonstrated excellent effectiveness and tolerability of ETN among Japanese patients with RA; however, additional subanalyses based on age, duration of RA, and Steinbrocker stage may reveal features that will allow targeting treatment for optimal safety and effectiveness.

Most AE observed during this study were not serious, and the incidence of serious AE (7.54%) was similar to that observed in clinical trials involving ETN (5%[16], 7%[17], and 11%[18]). This surveillance showed that the most commonly observed AE was injection site reactions (5.3%), and the most frequently observed serious AE was pneumonia (0.8%). The second most common serious AE was interstitial pneumonia. Multiple underlying factors can cause interstitial lung disease among RA patients, including underlying RA, concomitant use of DMARD, and opportunistic infection such as cytomegalovirus. However, there was no further information to identify the cause in this survey; additional investigation is required for further analysis. As in clinical trials, these results indicate that ETN was well tolerated for 24 weeks in Japanese patients treated in a real-world setting.

There have been reports about activation of latent TB by TNF inhibitors[19-22]. The incidence of TB in Japan is estimated at 28/100,000 and in the US at 5/100,000[23]. One of the key objectives of this PMS was to promote the appropriate screening and prophylaxis of TB for RA patients starting ETN because Japan is a region endemic for TB. The Japan College of Rheumatology published guidelines for screening for latent TB before initiation of TNF inhibitor and recommended the use of prophylactic INH if necessary[23].

The PMS interim results demonstrated that a very high percentage of ETN patients (98.5%) were screened for TB

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Table 5. Measures of effectiveness in patients participating in postmarketing surveillance of etanercept in Japan.

Effectiveness Measure, mean (SD)	Period After Treatment Initiation, weeks		
	Baseline	4*	24*
DAS28 score	6.0 (1.2)	4.4 (1.3)	3.9 (1.3)
n	4643	2511	4248
No. tender joints	9.9 (7.1)	4.5 (5.1)	3.3 (4.4)
n	6425	4010	6229
No. swollen joints	9.1 (6.3)	4.3 (4.5)	3.0 (3.7)
n	6425	4009	6227
Visual analog scale score, mm	60.9 (22.4)	37.2 (22.6)	31.3 (22.5)
n	5876	3420	5464
ESR, mm/h	61.1 (33.2)	39.3 (28.1)	37.1 (28.3)
n	5059	3024	4923
CRP, mg/dl	3.8 (3.4)	1.4 (2.1)	1.3 (2.2)
n	6399	4221	6427
Duration of morning stiffness, min	111.5 (181.7)	44.3 (114.4)	31.0 (90.3)
n	5797	3169	4988

CRP: C-reactive protein; DAS: Disease Activity Score; ESR: erythrocyte sedimentation rate. * All different compared to baseline, $p < 0.001$.

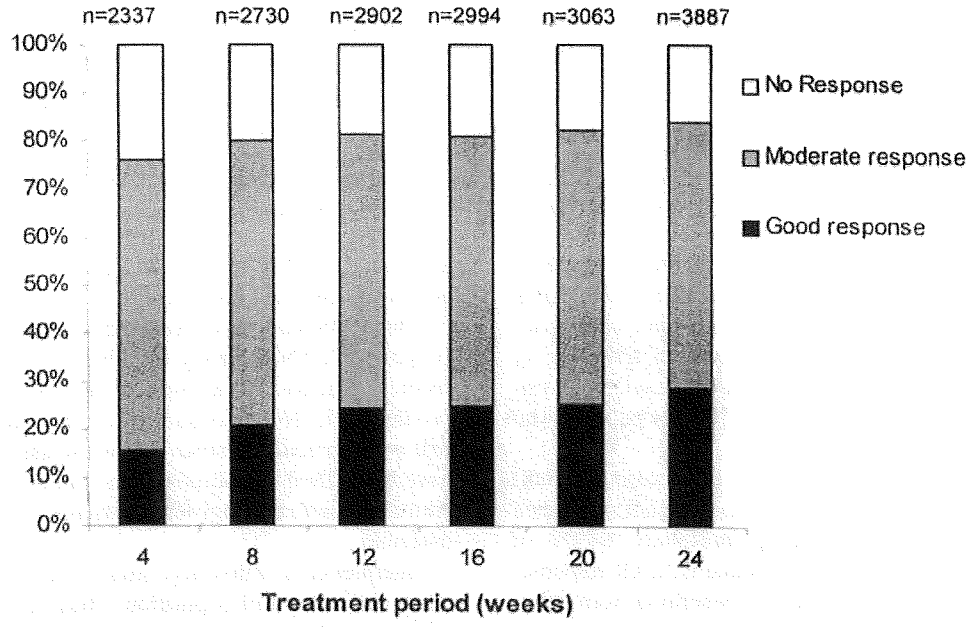

Figure 3. Effectiveness of etanercept in patients for the first 24 weeks as indicated by EULAR response rate, using DAS28 (last observation carried forward).

by chest radiograph. Ten cases, including 2 unconfirmed cases, were reported as TB in this PMS. Two cases developed TB despite the use of INH, with retrospective analysis suggesting active TB at onset of ETN. The remaining 6 confirmed cases did not receive prophylactic INH. In this interim analysis, 453 patients (6.5%) out of 7,099 cases had a medical history of TB, and 444 of them (98%) were treated with prophylactic INH. Reactivation of latent TB was not observed among patients who had appropriate screening and received prophylactic INH. The interim results of this PMS suggest that careful screening is important to avoid the use of TNF inhibitors in those with active TB, and that prophylactic INH appears to be effective when prescribed for suspected latent TB.

Increased risk of certain types of malignancy, such as lymphoma, is reported in patients with RA. However, the association of TNF inhibitors and risk of malignancy is still uncertain[24-26]. Although 14 cases of malignancy were reported during the 24-week observation period in this PMS, it is difficult to estimate if the number is high or low as there

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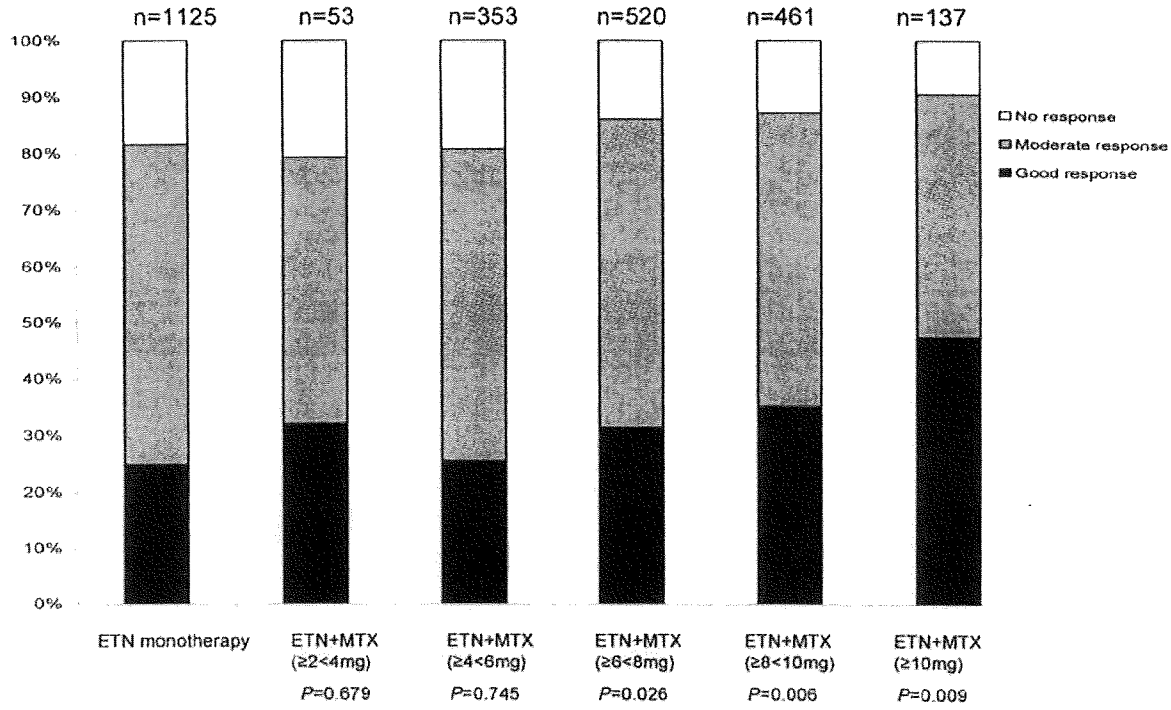

Figure 4. Effectiveness of etanercept (ETN) for the first 24 weeks in patients receiving ETN monotherapy or ETN plus methotrexate (MTX), as indicated by EULAR response criteria using DAS28 (last observation carried forward). Total number of ETN + MTX cases = 1,526. Two cases were ETN + MTX (< 2 mg/wk); one case was classified as good response, the other as moderate response. Response rate was compared with that of ETN monotherapy by chi-square test. Trend on response rate evaluated by Cochran-Armitage test; result was significant ($p < 0.001$).

is no appropriate control regarding the incidence of malignancy among Japanese with RA. It is also possible that malignancy might have already existed in some cases prior to initiation of ETN. Careful interview and screening of patients, if necessary, may provide information for physicians to evaluate risk and benefit of ETN for patients who are at risk of having malignancy.

Japanese guidelines for use of TNF inhibitor in RA indicate the use of biologics when patients have not responded to at least one DMARD. During this study, 84.1% of patients experienced a good or moderate EULAR response, a very positive result for patients not benefiting from previous treatments.

Patients who were prescribed higher doses of MTX concomitantly with ETN in this study showed a higher percentage of patients achieving a better response. Since the upper dose of MTX used in Japan is limited to 8 mg per week, much lower than that used in the US or Europe (5 mg to 20 mg per week), it may be possible that an even greater proportion of patients could achieve a good response if the MTX dose were increased.

From evaluation of 411 patients who had a history of infliximab use, 84.9% experienced a good or moderate EULAR response. This result suggests that, for patients who do not respond to or discontinue infliximab treatment, switching to ETN may be helpful in controlling disease activity, as reported elsewhere[23].

This PMS study was designed to evaluate the risks and benefits of ETN, a newly introduced product in Japanese medical practice. As a result of the excellent benefit/risk profile of ETN demonstrated in this interim report of real-world practice, restrictions on the use of ETN in Japan have been lifted. The final results will be evaluated for the entire population of registered patients when these data are available.

Interpretation of this large study is limited by the fact that there was no control population. Thus, it is difficult to discriminate patient outcomes caused by ETN from outcomes caused by other factors, such as the natural history of the disease, observer or patient expectations, or other treatments. Safety data were collected from all 7,091 cases, but there are missing data on effectiveness. Nonetheless, it does provide insight on the use of ETN in a large cohort of patients being treated in real-world settings.

This surveillance study has produced one of the largest observational databases available worldwide for patients with RA treated with biologics. It registered all patients with RA treated with ETN in Japan, and systematically gathered safety and effectiveness data. This interim analysis of over 7,000 patients provides useful data about real-world ETN

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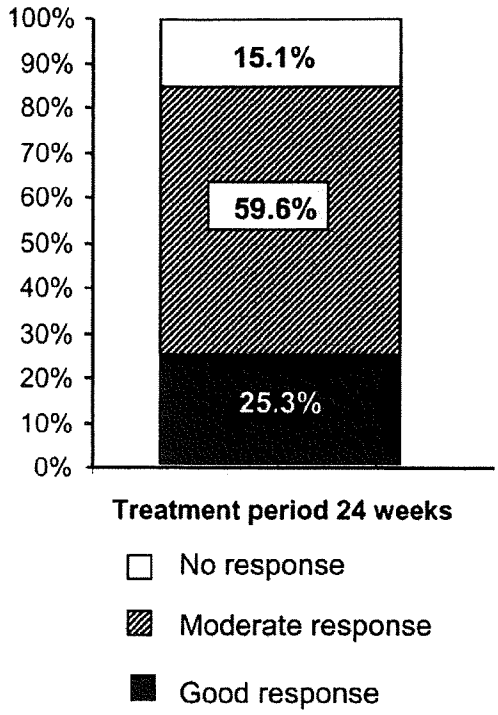

Figure 5. Effectiveness of etanercept for the first 24 weeks as indicated by response rate in patients switching from infliximab to etanercept (n = 411), based on EULAR response criteria using DAS28 (last observation carried forward).

use and supports the safety and effectiveness of ETN as a treatment for Japanese with RA. Additional analyses of data from this study may identify factors that influence safety and effectiveness of ETN and further assist in defining its appropriate use.

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