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Temporal Expression of Growth Factors Triggered by Epiregulin Regulates Inflammation Development

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In this study, we investigated the relationship between several growth factors and inflammation development. Serum concentrations of epiregulin, amphiregulin, betacellulin, TGF-α, fibroblast growth factor 2, placental growth factor (PLGF), and tenasin C were increased in rheumatoid arthritis patients. Furthermore, local blockades of these growth factors suppressed the development of cytokine-induced arthritis in mice by inhibiting chemokine and IL-6 expressions. We found that epiregulin expression was early and followed by the induction of other growth factors at different sites of the joints. The same growth factors then regulated the expression of epiregulin at later time points of the arthritis. These growth factors were increased in patients suffering from multiple sclerosis (MS) and also played a role in the development of an MS model, experimental autoimmune encephalomyelitis. The results suggest that the temporal expression of growth factors is involved in the inflammation development seen in several diseases, including rheumatoid arthritis and MS. Therefore, various growth factor pathways might be good therapeutic targets for various inflammatory diseases. The Journal of Immunology, 2015, 194: 1039–1046.

Interleukin-6 is a cytokine expressed by various activated cells, including CD4+ cells, and has an important role in the development of inflammation (1, 2). It is also required for the development of Th17 cells, which are IL-17–expressing activated CD4+ T cells (3), and strongly correlates with various inflammatory disease models (4). We previously identified the inflammation amplifier (formerly the IL-6 amplifier) as a fundamental mechanism of inflammation induction in such disease models as well as in human inflammatory diseases (4–6). The amplifier, which is activated by simultaneous stimulation of NF-κB and STAT3 via cytokines such as IL-17A and IL-6 in type 1 collagen* nonimmune cells, induces a positive feedback loop of IL-6 (5). The amplifier acts as a local chemokine inducer that accumulates various immune cells followed by the local dysregulation of homeostasis, that is, inflammation. Since its discovery, we have shown that the amplifier is hyperactivated by various factors, including cytokines, neurotransmitters, and the growth factor epiregulin (1, 4).

Growth factors consist of many groups, including the epidermal growth factor (EGF) family, the platelet-derived growth factor family, the vascular endothelial growth factor family, and the fibroblast growth factor (FGF) family, all of which have the potential to initiate and mediate many complex biological responses. Most receptors of these families have a tyrosine kinase region (7). The extracellular ligand–binding domain is more variable, leading to different ligand profiles even in the same receptor type. For example, ErbB1 (EGF receptor) binds to six members of a growth factor family that includes EGF, epiregulin, TGF-α, amphiregulin (Areg), and betacellulin (BTC). When bound by a ligand, ErbB1 is autophosphorylated at various cytoplasmic tyrosine residues, which creates docking sites for adaptor proteins followed by the

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Abbreviations used in this article: Areg, amphiregulin; BTC, betacellulin; EAE, experimental autoimmune encephalomyelitis; EGF, epidermal growth factor; FGF, fibroblast growth factor; HPRT, hypoxanthine phosphoribosyltransferase; MS, multiple sclerosis; PLGF, placental growth factor; RA, rheumatoid arthritis; shRNA, short hairpin RNA; TNC, tenasin C.

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activation of intracellular signaling cascades, including those of NF-κB, STAT3, and MAPK. Additionally, ErbB1 and its ligands have been shown to influence cellular growth and proliferation and are mainly associated with cancers and neoplasm processes. We recently showed that the epiregulin/ErbB1 axis contributes to activation of the inflammation amplifier and subsequent chronic inflammation development via the PI3Kα/NF-κB pathway. Furthermore, blocking the epiregulin/ErbB1 pathway suppresses several inflammatory disease models, whereas serum concentrations of epiregulin are higher in patients with inflammatory disease (4). In the present study, we investigated the relationship between other growth factors and local chemokine and IL-6 expression via the inflammation amplifier during the development of inflammation, mainly in an animal model of rheumatoid arthritis (RA).

In this study, we showed that the serum concentrations of several growth factors were increased in RA patients, whereas local blockades of each growth factor suppressed the development of cytokine-induced arthritis in mice by suppressing chemokine and IL-6 expressions. To understand why these growth factors act independently during the development of inflammation, we examined their temporal expression in joints. Only epiregulin was expressed by cytokine-mediated NF-κB and STAT3 activation. Epiregulin directly triggered the expression of other growth factors, although at the same time its expression was dependent on these growth factors at later time points of arthritis development. Consistent with this result, synovial cells expressed epiregulin by day 1 after cytokine injection, whereas the expression of other growth factors was observed at later times. Furthermore, elevated levels of various growth factors were detected in sera of patients suffering from multiple sclerosis (MS). Affected spinal cords in an MS model, experimental autoimmune encephalomyelitis (EAE), expressed most of the growth factors, and EAE symptoms were suppressed by the blockade of TGF-α. These results suggest that the temporal expression of growth factors triggered by the cytokine/epiregulin axis is independently involved in the development of various inflammatory diseases. Therefore, each growth factor pathway might be an independent therapeutic target for many inflammatory diseases, including RA and MS.

### Materials and Methods

#### Human serum preparations

Serum was collected from 11 patients with RA at Tokyo Medical and Dental University Hospital and from 21 patients with clinically defined MS (negative for autoantibody presence) at Osaka University Hospital. Serum was also collected from 41 healthy subjects at Osaka University Health Care Center. Informed consent was obtained from each subject. This study was approved by the Ethics Committees of Osaka University Hospital and Tokyo Medical and Dental University. Serum levels of Areg, BTC, TGF-α, FGF2, PLGF, and tenasin C (TNC) in patients were measured by a Milliplex kit from the Graduate School of Medicine, Osaka University.

#### In vivo neutralization and immunohistochemistry

The following Abs were used for in vivo neutralization and immunohistochemistry: monoclonal anti-mouse Areg Ab, anti-mouse BTC Ab, anti-mouse epiregulin Ab, anti-human TGF-α Ab, anti-mouse PLGF2 Ab, anti-human/mouse TNC Ab (R&D Systems, Minneapolis, MN), anti-mouse FGF2 Ab (Millipore, Tokyo, Japan), polyclonal anti-mouse epiregulin Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse FGF2 Ab (Abcam, Tokyo, Japan), and purified rat IgG (Sigma-Aldrich, Tokyo, Japan). The following Abs were used for Western blotting: anti-phospho-p65 (Ser536, 93H1), anti–phospho-Akt (Ser473, 1392H1), anti-Akt (all from Cell Signaling Technology, Tokyo, Japan), anti-p65 (C-20) (Santa Cruz Biotechnology), anti–α-tubulin (Sigma- Aldrich), HRP-conjugated goat anti-rabbit IgG (H+L) (SouthernBiotech, Birmingham, AL), and HRP-conjugated goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA). The following Abs were used for flow cytometry analysis: allopolycoyamin-conjugated anti–IFN-γ (BioScience, San Diego, CA) and control IgG1 (FITC-conjugated anti–CD8, BioScience), anti–CD11b (Beckman Coulter, Brea, CA), anti–CD11c (eBioscience), anti–CD19 (eBioscience), anti–NK1.1 (eBioscience), and anti–I–A/II–E (BioLegend, Tokyo, Japan); PE-conjugated anti–IL-17A (eBioLegend), control IgG2a (eBioLegend), and anti–I–A/II–E (BioLegend); and PE-Cy7–conjugated anti–CD4 (BioLegend).

#### Mission TRC short hairpin RNA (shRNA) clones, LPS, puromycin, polybrene, MOG, pertussis toxin, IFA, protease inhibitor mixture, phosphatase inhibitor mixture 2, phosphatase inhibitor mixture 3, and MTI (thiazolyl blue) were purchased from Sigma-Aldrich. Mouse Areg, BTC, epiregulin, FGF2, PLGF2, IL-23, human TGF-α, TNC, and soluble IL-6Rs were purchased from R&D Systems. Mouse IL-17 was purchased from PeproTech (Rocky Hill, NJ). Human IL-6 was purchased from Toray Industries (Tokyo, Japan). LY294002 was purchased from Merck.

#### Intra-articular injections (joint injections)

IL-17A (R&D Systems), IL-6 (Tory Industries), or saline were injected into the joints as described previously (10). Joints were injected with lentivirus carrying shRNA specific for Areg, BTC, Tgfa, Fgf2, Plgf2, TNC, and NF-κB p65 (RelA) (Sigma-Aldrich) or with a lentivirus carrying a scrambled sequence (Sigma-Aldrich) or anti-Areg, anti-BTC, anti–TGF-α, anti–FGF2, anti–PLGF2, and anti–TNC Abs.

#### Real-time PCRs

Total RNA was prepared from BC1 and MEF cells using a GenElute mammalian total RNA kit (Sigma-Aldrich) or prepared from synovial tissues of mouse knee joints using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), chloroform (Sigma-Aldrich), and isopropanol (Sigma-Aldrich). The RNA was then treated with DNase I (Sigma-Aldrich) and used for reverse transcription with Moloney murine leukemia virus reverse transcriptase (Promega, Tokyo, Japan). cDNA product was used in each real-time PCR reaction. A 7300 Fast real-time PCR system (Applied Biosystems, Tokyo, Japan) and SYBR Green PCR master mix (Kapa Biosystems, Woburn, MA) were used to quantify levels of target mRNA and hypoxanthine phosphoribosyltransferase (HPRT) mRNA. The PCR primer pairs were as follows (forward/reverse): mouse HPRT primers, 5'-GATTAGGCGATGATAGACCGATGTT-3' and 5'-CTCCCATCTCCTCTGCATGACA-3'; mouse IL-6 primers, 5'-GAGGATTACACTTCCACAGACC3' and 5'-AAGGCTACATCCTGTTCTGACA-3'; mouse BTC primers, 5'-GCAGTTTCCTTCTGTTGTCGAT-3' and 5'-GGGTTTGCTTTTTCTCCCTTGGA-3'; mouse E-cadherin primers, 5'-GGCAGATGAGACACCTCATCAG-3' and 5'-GGATTAGCGATGATAGACCGATGTT-3'; mouse FGF2 primers, 5'-CTGCTCTCTGTTGCCTGCTGTC-3' and 5'-GAGGATTACACTTCCACAGACC3'; mouse epiregulin primers, 5'-CTGCCTCTGTTGCCTGCTGTC-3' and 5'-GAGGATTACACTTCCACAGACC3'; mouse Areg primers, 5'-GAGCTCTCTCGGTGCTGTCGAT-3' and 5'-GGGTTTGCTTTTTCTCCCTTGGA-3'; mouse TNC primers, 5'-GGGATTACACTTCCACAGACC3' and 5'-GAGGATTACACTTCCACAGACC3'; mouse PLGF2 primers, 5'-GGGATTACACTTCCACAGACC3' and 5'-GAGGATTACACTTCCACAGACC3'. These primers were designed using the Primer3 software and were synthesized by Biosearch Technologies (Corte Madera, CA). Real-time PCRs were performed in 96-well plates with 10 μl of cDNA as a template, 5 μl of SYBR Green PCR master mix (Kapa Biosystems), and 5 μl of 20 μM primers in triplicate. The amplification program was as follows: 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s followed by 40 cycles at 60°C for 1 min. PCR efficiency was calculated using standard curves generated from several mices, which reduces the number of animals used. We confirmed that the quantitative PCR results were equivalent between ankle and knee samples.
Clinical assessment of arthritis

Mice were inspected and assessed for signs of arthritis as described previously (4, 8, 10). In brief, the severity of the arthritis was determined based on two bilaterally assessed parameters: 1) swelling in the ankle, and 2) restricted mobility of the ankle joints. The severity of each parameter was graded on a scale of 0–3: 0, no change; 1, mild change; 2, medium change; and 3, severe change. Averages for a single point in one leg ankle joint from each mouse were used. The disease phenotypes and the histology were scored blindly. In some experiments, we injected shRNA lentiviruses into the joints because we hypothesized that the shRNA lentiviruses would reduce target expressions due to their significant knockdown of genes in BC1 cells.

Cells and stimulation conditions

A type 1 collagen† endothelial cell line of BC1 cells was obtained from Dr. M. Miyasaka (Osaka University) (4). For stimulation, BC1 cells were plated in 96-well plates (1 × 10^4 cells/well) and stimulated with human IL-6 (50 ng/ml; Toray Industries) plus human soluble IL-GR (50 ng/ml; R&D Systems) and/or mouse IL-17A (50 ng/ml; R&D Systems) for 3 or 24 h after 2 h of serum starvation. Cell culture supernatant was collected for ELISA and cell growth was assessed by MTT assay. In some experiments, cells were harvested and total RNA was prepared for real-time PCRs.

ELISA

IL-6 concentrations in cell culture supernatant or serum were determined using ELISA kits (BD Biosciences).

MTT assay

Cell growth was determined with thiazolyl blue tetrazolium bromide (Sigma-Aldrich) according to the manufacturer’s instructions.

Western blotting

BC1 cells were stimulated by the indicated cytokines and lysed with lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) supplemented with protease inhibitor mixture, phosphatase inhibitor mixture 2 (Sigma-Aldrich), and phosphatase inhibitor mixture 3 (Sigma-Aldrich). Twenty micrograms total protein was run on 5–20% SDS-PAGE (Wako, Tokyo, Japan). After transfer to a polyvinylidene fluoride membrane (Millipore), immunoblotting was performed according to the manufacturer’s protocol.

Luciferase reporter assay

Ankle joints from NF-κB-reporter Tg/F759 mice were collected, and synovial tissues were homogenized in passive lysis buffer (Promega). After centrifugation, the supernatants were collected, and total protein amount was adjusted by the Bradford method. Luciferase activities of tissue lysates were measured using a luciferase reporter assay system (Promega).

Histological analysis

Ankle joints were fixed in 4% paraformaldehyde, decalcified for 12 h in Morse’s solution (22.5% bornyl formate and 10% sodium acid citrate solution) followed by 12 h in 4% paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin, anti–phospho-STAT3, anti–phospho-EGFR (Cell Signaling Technology), anti–phospho-p65, anti-vimentin (Sigma-Aldrich), anti–type 1 collagen (Abcam), anti-Areg, anti–FGF2 Ab, anti–TGF-α Ab, and anti-epiregulin Ab (10).

Passive transfer of pathogenic CD4+ T cells from mice to induce EAE

EAE induction was performed as described previously (5, 11). Briefly, C57BL/6 mice or C57BL/6-PL mice were injected with a MOG35-55 peptide (Sigma-Aldrich) in CFA (Sigma-Aldrich) at the base of the tail. Day 0 followed by i.v. injection of pertussis toxin (Sigma-Aldrich) on days 0, 2, and 7. On day 9, CD4+ T cells from the resulting mice were sorted using anti-CD4 microbeads (Milteny Biotec, Tokyo, Japan). The resulting CD4+ T cell-enriched population (4 × 10^6 cells) was cocultured with IL-23 (10 ng/ml; R&D Systems) in the presence of MOG peptide-pulsed irradiated splenocytes (1 × 10^6 cells) for 2 d. Cells (1.5 × 10^7 cells) were then injected i.v. into wild-type mice. Clinical scores were measured as described previously (5, 11).

Mononuclear cell isolation from spinal cords

Mononuclear cells were isolated from spinal cords after cardiac perfusion with PBS, as described previously (11).

Intracellular cytokine staining

The number of Th17 cells in vivo was determined as described (12). In brief, T cells from spinal cords were stimulated with PMA and ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for 6 h. Intracellular IL-17 and IFN-γ were labeled with anti-IL-17 and anti–IFN-γ Abs, respectively, after surface staining, fixation, and permeabilization.

Flow cytometry

For cell surface labeling, 10^6 cells were incubated with fluorescence-conjugated Abs for 30 min on ice. The cells then were analyzed with a CyAn flow cytometer (Beckman Coulter, Tokyo, Japan). The collected data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis

Student t tests (two-tailed) and a Williams’ test were used for statistical analyses of differences between two groups. One-way ANOVA with a Dunnett post hoc analysis was used for multiple comparisons. A Wilcoxon rank-sum test was used for the statistical analyses of serum growth factor levels in humans (Figs. 1A, 4A) and clinical scores of arthritis and EAE (Fig. 1C, 1D, Supplemental Fig. 4B). A p value <0.05 was considered statistically significant.

Results

Various growth factors were increased in patients with RA

We previously showed that sera from patients suffering from RA have higher concentrations of epiregulin than do sera from control subjects (4). Also higher were the growth factors Areg, BTC, TGF-α, PLGF, TNC, and FGF2 (Fig. 1A). IL-6 concentration was also increased in sera from RA patients, as reported previously (4). These results suggest that various growth factors are involved in the development of inflammation.

Various growth factor pathways are critical for the development of a mouse RA model, F759 arthritis

We next investigated whether growth factors contribute to the development of arthritis in an RA model, F759 mice, which show spontaneous development of an arthritis that resembles human RA. These mice express a mutant variant of the IL-6 signaling transducer gp130 (Y759F) and have an enhanced IL-6–mediated STAT3 pathway due to deficient SOCS3-mediated negative feedback (8, 13). As these mice age, they spontaneously develop an MHC class II–associated, IL-6–dependent joint disease (F759 arthritis) that resembles RA (8, 14). Direct intra-articular injections (joint injections) of IL-17A and IL-6 with a minimum modification of hematopoietic cells induced arthritis within 2 wk in a manner dependent on NF-κB and STAT3 in nonimmune cells (10).

Joint injections of IL-17A and IL-6 increased the expressions of Areg, BTC, TGF-α, PLGF2 (mouse PLGF), TNC, and FGF2, as well as IL-6 in the joints (Fig. 1B). It was reported that EGF sometimes suppresses E-cadherin to induce epithelial–mesenchymal transition (15). We found that samples with increased TGF-α expression after IL-17 and IL-6 stimulation suppressed the expression of E-cadherin and had comparable expressions of β-actin to a control sample without cytokine stimulation (Supplemental Fig. 1A). We also confirmed that some growth factors were increased in other RA models such as collagen-induced arthritis and collagen Ab-induced arthritis (Supplemental Fig. 1B, 1C).

Importantly, joint injections of Abs against these growth factors or lentiviruses that had corresponding shRNA suppressed the development of the cytokine-induced arthritis (Fig. 1C, 1D). Furthermore, blockades of each growth factor decreased the expressions of IL-6 and CCL20 (Fig. 1E, 1F), which are essential for the development of arthritis (4, 10). These results suggest that growth factor pathways are independently involved in the development of cytokine-induced arthritis in vivo.
Areg, BTC, TGF-α, FGF2, PLGF, and TNC play a role in the hyperexpression of IL-6 and chemokines via the PI3K/NF-κB pathway

We next identified which cell types produce and respond to growth factors in F759 arthritis. Immunohistochemistry experiments showed that 75% of observed cells had phosphorylated EGFR in the joints with cytokines and a similar percentage of cells had phosphorylated STAT3 and NF-κB and expressed various growth factors, including Areg, epiregulin, TGF-α, and FGF2 (Fig. 2A).

**FIGURE 1.** Areg, BTC, TGF-α, FGF2, PLGF, and TNC are critical for the development of cytokine-induced arthritis. (A) Serum concentrations of Areg, BTC, TGF-α, FGF2, PLGF, and TNC in patients suffering from RA ($n = 11$) compared with healthy age- and sex-matched subjects ($n = 26$). (B) IL-17A (0.2 μg) and IL-6 (0.2 μg) on days 0, 1, and 2 were injected into the knee joints of F759 mice. mRNA expressions of Areg, Btc, Tgf-α, Fgf2, Plgf2, and TNC in joint synovial tissues were analyzed on day 7. (C) Clinical arthritis scores from the left legs of F759 mice after left ankle joint injections of 0.1 μg IL-6 and IL-17 on days 0, 1, and 2 and joint injections of anti-Areg Abs (1 μg, $n = 6$), anti-Btc Abs (1 μg, $n = 6$), anti–TGF-α Abs (1 μg, $n = 6$), anti-FGF2 Abs (1 μg, $n = 6$), anti-PLGF2 Abs (1 μg, $n = 6$), anti-TNC Abs (1 μg, $n = 6$), anti-IgG (1 μg, $n = 6$), or PBS with neither IL-6 nor IL-17 (n = 6) once every 2 or 3 d for 0–22 d. (D) Clinical arthritis scores from the left legs of F759 mice after left ankle joint injections of 0.1 μg IL-6 and IL-17 on days 6, 7, and 8 and joint injections of lentivirus encoding shRNA specific for p65 NF-κB (RelA) (1.9 $\times$ 10$^5$ transducing units [TU], $n = 6$), Areg (1.9 $\times$ 10$^5$ TU, $n = 6$), BTC (1.9 $\times$ 10$^5$ TU, $n = 6$), Tgfa (1.9 $\times$ 10$^5$ TU, $n = 6$), Fgf2 (1.9 $\times$ 10$^5$ TU, $n = 6$), Plgf2 (1.9 $\times$ 10$^5$ TU, $n = 6$), TNC (1.9 $\times$ 10$^5$ TU, $n = 6$), a nontarget sequence (1.9 $\times$ 10$^5$ TU, $n = 6$), or saline with neither IL-6 nor IL-17 (n = 6) on days 0, 2, and 4 during days 0–29. (E and F) IL-17 (0.2 μg) and IL-6 (0.2 μg) on days 0, 1, and 2 were injected into the knee joints of F759 mice in the presence or absence of joint injections of anti-Areg Ab (1 μg, $n = 12$), anti-BTC Ab (1 μg, $n = 12$), anti–TGF-α Ab (1 μg, $n = 12$), anti-FGF2 Ab (1 μg, $n = 12$), anti-PLGF Ab (1 μg, $n = 12$), control IgG (1 μg, $n = 12$) on days 0, 1, 2, 4, and 6 followed by analysis of expressions of IL-6 (E) and CCL20 (F) in joint synovial tissues on day 7. Individual values, mean scores ($A$), and mean scores ± SEM ($B$–$F$) are shown. The $p$ values were calculated using a Wilcoxon test ($A$, $C$, and $D$), Student $t$ test ($B$), and one-way ANOVA ($E$ and $F$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; *$p < 0.05$ versus each treatment group in ($C$) and ($D$). *$p < 0.05$ versus sh-Fgf2, sh-Areg, sh-Btc, and sh-Tnc in ($D$).
Chemokines and IL-6 expressions were significantly reduced in cultures without FBS, a rich source of growth factors, despite stimulation with IL-17A and/or IL-6 (Supplemental Fig. 2A) (4). We then obtained recombinant molecules of each growth factor. All except PLGF2 and TNC enhanced the expression of chemokines and IL-6, but those of PLGF2 and TNC are not.

It is important to understand how growth factors affect NF-κB and/or STAT3 signaling, and thus the inflammation amplifier. A PI3K inhibitor, LY294002, but not an MEK inhibitor, suppressed growth factor–mediated IL-6 expression (Fig. 2E, Supplemental Fig. 2B). Furthermore, Areg, BTC, TGF-α, and FGF2 enhanced the phosphorylation of Akt and p65 NF-κB in vitro and the activity of a NF-κB reporter in the presence of IL-17A and IL-6 in vivo (Fig. 2F, 2G). To confirm the importance of PI3K for growth factor–mediated IL-6 expression, we employed wortmannin and RNA interference. We used shRNA of PI3Kα because we previously reported epiregulin-EGFR enhances IL-6 expression via PI3Kα in the presence of IL-17 and IL-6 (4). Wortmannin and shRNA of PI3Kα suppressed growth factor–mediated IL-6 expression (Supplemental Fig. 2C, 2E), which demonstrates that PI3K, particularly PI3Kα, is critical for the growth factor–mediated en-

**FIGURE 2.** Areg, BTC, TGF-α, and FGF2 enhance the expressions of IL-6 and chemokines via the PI3K/NF-κB axis. (A) IL-6 (1 μg) and IL-17 (1 μg) were injected into the left ankle joints of F759 mice on days 0, 1, and 2. Immunohistochemistry of the left ankle joints was performed by using Abs against Areg, epiregulin (Ereg), FGF2, TGF-α, p-STAT3, p-p65, p-EGFR, type 1 collagen, and vimentin on day 7. These experiments were performed at least three times independently. Frequency of cells that showed activation of the inflammation amplifier (p-STAT3+p-p65+), received EGFR signaling (p-EGFR+), or produced growth factors (Areg+Ereg+FGF2+TGF-α+) is indicated. Col1, type 1 collagen; Vim, vimentin. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test). (B) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα and/or mouse IL-17 for 24 h with or without Areg, BTC, TGF-α, FGF2, PLGF2, and TNC. Culture supernatants were collected and assessed using ELISA specific for IL-6. Samples without growth factors (filled columns) were compared with samples with each growth factor. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA). (C) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα and mouse IL-17 for 3 h after stimulation with human IL-6 plus soluble IL-6Rα and mouse IL-17 with or without Areg, BTC, TGF-α, and FGF2. mRNA expressions of IL-6 and CCL20 were evaluated using real-time PCR. Samples without growth factors (filled columns) were compared with samples with each growth factor. *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA). (D) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα and mouse IL-17 for 24 h with or without 0.5 h pretreatment of LY294002 (3 μM) or DMSO vehicle control. Culture supernatants were collected and assessed using ELISA specific for IL-6. Cell survival was evaluated based on mitochondrial activity. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test). (E) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα and mouse IL-17 in the presence or absence of Areg, BTC, TGF-α, or FGF2 for 30 min and then investigated for the phosphorylation of Akt and p65. (F) IL-6 and IL-17 were injected into the ankle joints of NF-κB reporter Tg/F759 mice with or without 0.2 μg Areg, BTC, TGF-α, or FGF2 followed by analysis of NF-κB reporter activity in the ankle joints on day 7 using the luciferase reporter assay system. *p < 0.05 (one-way ANOVA). Mean scores ± SD (A–E) and mean scores ± SEM (G) are shown.
hancement of inflammation. We also found that some growth factors enhance IL-6 expression in the presence of IL-17 and IL-6 in primary synovial fibroblasts in a manner dependent on PI3K (Supplemental Fig. 3A, 3B). Alternatively, PLGF2 and TNC increased cellular proliferation (Supplemental Fig. 3C). These results strongly suggest that most of the examined growth factors enhanced the PI3K/NF-κB pathway to increase the expression of chemokines and IL-6, whereas the roles of PLGF2 and TNC might locally increase cell growth to increase the number of cells involved in inflammation at diseased sites such as the joints.

**Growth factor expressions are regulated in an epiregulin-triggered temporal manner**

We next investigated why the growth factors act independently and with no compensation mechanisms for the development of cytokine-induced arthritis. IL-17A and IL-6, which are the triggering cytokines for inflammation development, increased epiregulin, but not the expression of the other growth factors in vitro (Fig. 3A). At the same time, epiregulin induced the expression of the other growth factors (Fig. 3B), probably after the development of inflammation. Consistent with this thought, joint injections of IL-17A and IL-6, which induce arthritis, increased epiregulin rapidly and intensely compared with other growth factors (Fig. 3C). Furthermore, the expressions of the other growth factors were suppressed in the presence of an epiregulin-neutralizing Ab even after joint injections of IL-17A and IL-6 (Fig. 3D, Supplemental Fig. 4A). Alternatively, blockade of each growth factor also suppressed epiregulin expression at later time points of the arthritis development (Fig. 3E), suggesting a reciprocal regulation mechanism between growth factors for the maintenance of epiregulin.

That epiregulin triggers a temporal expression of growth factors was also confirmed by immunohistochemistry. The expression of

**FIGURE 3.** Presence of epiregulin-triggered temporal expressions in affected tissues of cytokine-induced arthritis. (A) mRNA expressions of Areg, Btc, Tgfa, epiregulin (Ereg), Fgf2, Plgf2, and Tnc in BC1 cells in the presence or absence of stimulation with IL-17 and IL-6 were evaluated 3 h later using real-time PCR. (B) mRNA expressions of Areg, Btc, Tgfa, Ereg, Fgf2, Plgf2, and Tnc in BC1 cells in the presence or absence of epiregulin stimulation were evaluated 3 h later using real-time PCR. (C) IL-17 (0.2 μg) and IL-6 (0.2 μg) on days 0, 1, and 2 were injected into the knee joints of F759 mice followed by analysis of expressions of Ereg, Areg, and TGF-α in joint synovial tissues on days 0, 1, 3, 5, and 7 (n = 3 for each condition). (D and E) IL-17 (0.2 μg) and IL-6 (0.2 μg) on days 0, 1, and 2 were injected into the knee joints of F759 mice followed by analysis of expressions of Ereg, Areg, TGF-α, FGF2, Plgf2, and Tnc in joint synovial tissues on days 0, 1, 3, 5, and 7 (n = 3 for each condition). (F and G) IL-6 (1 μg) and IL-17 (1 μg) on days 0, 1, and 2 were injected into the left ankle joints of F759 mice followed staining by using antibodies against Ereg, TGF-α, and FGF2 in paraffin sections of left ankle joints on days 1 (F) and 7 (H) by immunohistochemistry. These experiments were performed at least three times independently; representative data are shown. Arrows indicate cells expressing growth factors in the ankle joint synovial tissues. Scale bars, 100 μm. Quantification of the histological analysis (10 × 0.1 mm² field) for (F) and (H) is shown (G and I). Mean scores 6 SD (A–E) and mean scores 6 SEM (G and I) are shown. The p values were calculated using a Student t test (A and B) and one-way ANOVA (D and E). *p < 0.05, **p < 0.01, ***p < 0.001.
epiregulin, but not TGF-α or FGF2, was observed in the joints 1 d after IL-17A and IL-6 cytokine injections (Fig. 3F, 3G). The expressions of epiregulin and TGF-α were broad in the joints by day 7 after cytokine injections, whereas those of FGF2 were restricted to the middle and distal areas (Fig. 3H, 3I). Thus, growth factor expressions are regulated in an epiregulin-triggered temporal manner in the affected joints of F759 arthritis.

Growth factors are increased in patients with MS and are critical for the development of an MS model, EAE

We also investigated roles of growth factor expressions during the development of other autoimmune diseases. We found that Areg, BTC, TGF-α, FGF2, PLGF, and TNC were increased in sera of patients with MS (Fig. 4A), consistent with sera from patients suffering from MS having higher concentrations of epiregulin than sera from control subjects (4). We further investigated the growth factors in an MS model, EAE. The expressions of growth factors increased in the L5 cord where pathogenic CD4+ T cells are initially accumulated (Fig. 4B), suggesting that the growth factors are involved in the development of EAE. Importantly, administrations of anti–TGF-α Ab or anti-epiregulin Ab significantly suppressed the development of EAE (Fig. 4C, Supplemental Fig. 4B). Serum IL-6 and the number of infiltrating CD4+ cells with IL-17 or IFN-γ in the L5 cord were also decreased after treatment of anti–TGF-α Ab (Fig. 4D, 4E). Additionally, we constantly detected low cell numbers in the spinal cords after EAE induction where we previously reported similar numbers of T cells (4). These results support the idea that the regulation of growth factors contributes to the development of inflammation in other autoimmune diseases such as MS.

Discussion

We recently showed that the epiregulin/ErbB1 axis is involved in the development of inflammation in an RA model, an MS model, and a chronic rejection model (4, 16). In this study, we show that serum concentrations of growth factors including not only epiregulin, but also Areg, BTC, TGF-α, FGF2, PLGF, and TNC, increase in RA patients, suggesting that various growth factors are involved in RA development. Indeed, joint injections of IL-17A and IL-6, which induce arthritis in F759 mice, increased the local expression of these growth factors. At the same time, blockades of these factors suppressed the development of cytokine-induced arthritis in F759 mice. Moreover, we showed that many growth factors such as epiregulin, Areg, BTC, TGF-α, and FGF2 were increased in sera of patients suffering from MS and in the L5 cord of EAE, an MS model, and that blockade of TGF-α or epiregulin suppressed the development of EAE (Fig. 4C, Supplemental Fig. 4B). These results suggest that various growth factors might be independent therapeutic targets for various inflammatory diseases, including RA and MS.

FIGURE 4. Growth factors are critical for the development of an MS model, EAE. (A) Serum concentrations of Areg, BTC, TGF-α, FGF2, PLGF, and TNC in patients suffering from MS (n = 21) compared with healthy age- and sex-matched subjects (n = 15). (B) mRNA expressions of epiregulin (Ereg), Areg, Btc, TGF-α, FGF2, PLGF, and TNC in the L5 cord 7 d after transfer of pathogenic CD4+ T cells were evaluated using real-time PCR. (C–E) Pathogenic CD4+ T cells isolated from L5 spinal cords of Th17-transferred C57BL/6 mice were isolated on day 10. The resulting cell populations were counted and stimulated in vitro with MOG peptide and bone marrow–derived dendritic cells. Twenty-four hours after in vitro stimulation, intracellular IL-17 and IFN-γ levels were examined. The numbers of CD4+IL-17+ and CD4+IFN-γ+ T cells in spinal cords were significantly lower in recipients treated with anti–TGF-α Ab (n = 5) than in those treated with control IgG (n = 5). Individual scores, mean scores (A), and mean scores ± SEM (B–E) are shown. The p values were calculated using Wilcoxon tests (A and C) and Student t test (B, D, and E). *p < 0.05, **p < 0.01, ***p < 0.001.
We also analyzed the molecular mechanism for how these growth factors work independently to develop inflammation. We first investigated the expressions of these growth factors in affected joints during the course of the arthritis development. Only epiregulin was induced at the early phase of the inflammation, but other growth factors showed increased expression in the joints at the late phase. Furthermore, epiregulin expression itself was also dependent on the expression of each growth factor during the late phase of inflammation. Consistent with these in vivo results, in vitro experiments showed that IL-17A and IL-6 increased epiregulin expression but not other growth factors, but that epiregulin increased the expression of other growth factors. These results strongly suggest epiregulin-triggered temporal expression of growth factors in the affected tissues, which induces reciprocal regulation of the growth factors, is involved in the development of inflammation during cytokine-induced arthritis. Thus, one explanation for why growth factors work independently to develop inflammation is their temporal regulation in the affected tissues.

Interestingly, there are two kinds of growth factors that contribute to inflammation development. One group includes factors that enhance activation of the inflammation amplifier, such as epiregulin, Areg, BTC, TGF-α, and FGF2. These factors enhance activation of the inflammation amplifier via the PI3K/NF-κB pathway. The second group includes PLGF2 and TNC, which increase cell proliferation. We hypothesize that the increased cell numbers by PLGF2 and TNC enhanced the activation of the inflammation amplifier, because various growth factors and cytokines, including NF-κB and STAT3 stimulators, surround the fibroblasts to enhance proliferation. Moreover, the affected tissues in EAE contained various growth factors, including PLGF2 and TNC. Thus, we propose that a temporal expression of growth factors regulates the expression of chemokines and the proliferation of nonimmune cells, both of which contribute to inflammation in the joints of F759 mice as well as the CNS of EAE.

In summary, we investigated the relationship between growth factors and inflammation. Most growth factors tested induced IL-6 and chemokine expressions via the PI3K/NF-κB pathway. Furthermore, regional blockades of the growth factors suppressed the development of cytokine-induced arthritis. Moreover, these growth factors increased in sera of patients suffering from RA. These results suggest that each growth factor independently plays a critical role in RA development even though most of them activate similar signaling pathways. We also revealed important aspects of the molecular mechanism responsible, as epiregulin-triggered temporal regulation of the growth factors contributed to the development of inflammation, and each growth factor reciprocally regulated epiregulin in the affected tissue during the late phase of the disease development. Importantly, various growth factors increased in patients with MS and are involved in the development of EAE. We therefore conclude that these growth factors might be therapeutic targets for various inflammatory diseases, including RA and MS.

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Disclosures
The authors have no financial conflicts of interest.

References
Fig S1. E-cadherin and β-actin levels after IL-6 and IL-17 treatment in the joints and the upregulation of growth factors and IL-6 in commonly used arthritis models.

(A) IL-17 (0.2 µg) and IL-6 (0.2 µg) were injected into the knee joints of F759 mice on days 0, 1 and 2. TGFα, E-cadherin and β-actin mRNA levels in joint synovial tissues were examined on day 7.

(B) Collagen antibody-induced arthritis (CAIA) in DBA/1J mice. Saline or 1.5 mg of arthrogenic monoclonal antibody cocktail (Ab, Chondrex, Inc.) was injected i.v. on day 0. LPS (50 µg) was injected i.p. on day 3. IL-6 and growth factor mRNA levels in knee joint tissues were examined by qPCR on day 5.

(C) Collagen-induced arthritis (CIA) in DBA/1J mice. Mice were immunized s.c. with chicken type II collagen emulsified with CFA on days 0 and 21. IL-6 and growth factor mRNA levels in knee joint tissues were examined by qPCR on day 30. Normal mice were left untreated. Mean scores + SEM are shown. p values were calculated using Student’s t tests (*p < 0.05, **p < 0.01, *** p < 0.001 and NS, not significant).
**Fig S2.** Bovine serum and growth factors enhance IL-6 production in BC1 cells, which is mediated by PI3K pathway, but not MEK pathway.

(A) BC1 cells were starved for 2 hrs, followed by stimulation with human IL-6 plus soluble IL-6 receptor and mouse IL-17 in the presence or absence of 10% fetal bovine serum (FBS). Mouse IL-6 levels in the supernatant were measured 1 day later. Cell viability was evaluated based on mitochondria activity. Data represent mean + SD. *** p < 0.001 (Student’s t-test).

(B) BC1 cells were stimulated with IL-17 and human IL-6 plus soluble IL-6 receptor in the presence or absence of the indicated growth factors. MEK inhibitor PD98059 (B) or PI3K inhibitor wortmannin (C) was added to some cultures at the indicated concentrations. Culture supernatants were collected 1 day later and assessed using ELISA specific for mouse IL-6. Cell survival was evaluated based on mitochondrial activity. Data indicate mean + SD. # p < 0.05 and ### p < 0.001 vs. IL-6 + IL-17 without inhibitor (closed column, 0 µM), and * p < 0.05 vs. each growth factor without inhibitor (ANOVA).

(D) BC1 cells were infected with a lentivirus expressing shRNA against Pik3ca (PI3K catalytic subunit α) or non-target control shRNA (NTC). After puromycin selection, these BC1 cells were stimulated with IL-17 and human IL-6 plus soluble IL-6 receptor in the presence or absence of the indicated growth factors. Mouse IL-6 levels were measured 1 day later. Cell survival was evaluated based on mitochondrial activity. KD, knockdown. Data indicate mean + SD. *** p < 0.001 (Student’s t-test).

(E) The knockdown efficiency of Pik3ca evaluated by qPCR. Data indicate mean + SD. *** p < 0.001 (Student’s t-test).
Fig S3. Effect of growth factors on IL-6 production in primary synovial fibroblasts and cell growth in BC1 cells.

(A and B) Primary synovial fibroblasts were prepared from the ankle joints of C57BL/6 mice, as reported previously (Armaka M et al. Protocol Exchange doi:10.1038/nprot.2009.102 (2009)). (A) Synovial fibroblasts were stimulated with IL-17, human IL-6 plus soluble IL-6 receptor, or a combination of the two in the presence or absence of the indicated growth factors. Culture supernatants were collected 1 day later and assessed using ELISA specific for mouse IL-6. Cell survival was evaluated based on mitochondrial activity. Areg, amphiregulin; BTC, betacellulin. Data indicate mean + SD. * p < 0.05 and *** p < 0.001 vs. stimulation with IL-6 + IL-17 alone (ANOVA). (B) Synovial fibroblasts were stimulated with IL-17 and human IL-6 plus soluble IL-6 receptor in the presence or absence of Areg. Wortmannin was added at 1 µM to some cultures. Mouse IL-6 levels were measured 1 day later. Data indicate mean + SD. * p < 0.05 and NS, not significant (Student's t-test). (C) BC1 cells were stimulated with various concentrations of PLGF2 (left) and Tenascin C (TNC) (right) for 24 hr followed by assessment of cell growth by MTT assay. Mean scores ± SD are shown. p values were calculated using ANOVA (* p < 0.05 vs without growth factor).
Fig S4. Effect of anti-epiregulin on the expressions of growth factors in the joint and the development of EAE.

(A) IL-6 (0.2 µg) and IL-17 (0.2 µg) were injected on days 0, 1, and 2 into the knee joints of F759 mice with joint injections of anti-epiregulin antibody (1µg, n = 4, open circles) or control IgG (1µg, n = 4, black circles) on days 0, 1, 2, 3, and 5, followed by analysis of amphiregulin (Areg) (left) and TGFα (right) expressions in joint synovial tissues. Data indicate mean + SEM. * p < 0.05 (Student’s t-test) (B) Pathogenic CD4+ T cells isolated from EAE mice were intravenously transferred into wild-type C57BL/6 mice on day 0. Anti-Epiregulin antibody or control IgG (100 µg) was injected i.p. on days 0, 4, and 7. Clinical scores of EAE are shown (n = 3 for each group). Data indicate mean + SEM. * p < 0.05 (Wilcoxon test).