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Temporal Expression of Growth Factors Triggered by Epiregulin Regulates Inflammation Development

Masaya Harada,*,†,‡,§,† Daisuke Kamimura,*,†,‡,§,† Yasunobu Arima,*,†,‡,§ Hitoshi Kohsaka,† Yuji Nakatsuji,‖ Makoto Nishida,‡,§ Toru Atsumi,*,†,‡,§,† Jie Meng,*,†,‡,§ Hidenori Bando,*,†,‡,§ Rajeev Singh,* Lavannya Sabharwal,*,†,‡,§ Jing-Jing Jiang,*,†,‡,§ Norirko Kumai,*,†,‡,§ Nobuyuki Miyasaka,† Saburo Sakoda,** Keiko Yamauchi-Takihara,† Hideki Ogura,*,†,‡,§ Toshio Hirano,†† and Masaaki Murakami*,†,‡,§

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 immune cells followed by the local dysregulation of homeostasis, that is autophosphorylated at various cytoplasmic tyrosine residues, which creates docking sites for adaptor proteins followed by 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

*Division of Molecular Neuroimmunology, Institute for Genetic Medicine and Graduate School of Medicine, Hokkaido University, Sapporo 060-0815, Japan; †Laboratory of Developmental Immunology, Japan Science and Technology Agency–Core Research for Engineering, Science, and Technology, Graduate School of Frontier Biosciences, Osaka University, Osaka 565-0871, Japan; ‡Laboratory of Developmental Immunology, Japan Science and Technology Agency–Core Research for Engineering, Science, and Technology, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan; §Laboratory of Developmental Immunology, Japan Science and Technology Agency–Core Research for Engineering, Science, and Technology, World Premier International Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan; ‖Department of Medicine and Rheumatology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; ††Department of Neurology, National Hospital Organization Toneyama Hospital, Osaka 560-0045, Japan; and **Department of Neurology, National Hospital Organization Toneyama Hospital, Osaka 560-0045, Japan; and †††Japan Science and Technology Agency–Core Research for Engineering, Science, and Technology, Osaka University, 565-0871, Japan

‡M.H. and D.K. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Masaaki Murakami, Division of Molecular Neuroimmunology, Institute for Genetic Medicine and Graduate School of Medicine, Hokkaido University, Kita 15, Nishi 7, Kitaku, Sapporo 060-0815, Japan. E-mail address: murakami@igm.hokudai.ac.jp

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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 PI3K/Akt/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 Committee of Osaka University Hospital and Tokyo Medical and Dental University. Serum levels of Apc, B, TGF-α, FGF2, PLGF, and tenasin C (TNC) in patients were measured by a Milliplex kit from Bio-Rad Laboratories, Tokyo, Japan, or by use of a Luminex platform at the Graduate School of Frontier Biosciences and the Graduate School of Medicine, Osaka University.

Mouse strains

C57BL/6 and DBA/1J mice were purchased from Japan CLEA (Tokyo, Japan) or Japan SLC (Shizuoka, Japan). F759 mice, which carry a human gp130 variant (S710L), were backcrossed with C57BL/6 mice for >10 generations (8). NF-κB reporter transgenic mice in a C57BL/6 background were backcrossed with F759 mice and used for experiments (9). All mice were maintained under specific pathogen-free conditions according to the guidelines of the Institutional Animal Care and Use Committees of the Graduate School of Frontier Biosciences and the Graduate School of Medicine, Osaka University.

Abs and reagents

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 (Ser529, 951H1), anti-phospho-Akt (Ser473, Invitrogen, Carlsbad, CA), 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: allotype-specific conjugated anti–IFN-γ (eBioscience, San Diego, CA) and control IgG1 (eBioscience), FITC-conjugated anti–CD8 (eBioscience), anti–CD11b (Beckman Coulter, Breca, CA), anti–CD11c (eBioscience), anti-NK1.1 (eBioscience), and anti-I–A/I–E (BioLegend, Tokyo, Japan); PE-conjugated anti–IL-17A (eBioscience), control IgG2a (eBioscience), and anti–I–A/I–E (BioLegend); and PE-Cy7–conjugated anti–CD4 (BioLegend).

Mission TRC short hairpin RNA (shRNA) clones, LPS, puromycin, polybrene, MOG33–55, pertussis toxoid, IFNA, protease inhibitor mixture, phosphatase inhibitor mixture 2, phosphatase inhibitor mixture 3, and MTT (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). LV294002 was purchased from Merck.

Intra-articular injections (joint injections)

IL-17A (R&D Systems), IL-6 (Toray Industries), or saline were injected into the joints as described previously (10). Joints were injected with lentivirus carrying shRNA specific for Areg, BTC, Tgfα, Fgf2, Ptgf2, 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) was used. For some experiments, the ankles were used because they provide an easier assessment of restricted mobility by inflammation than do knee joints. For real-time PCRs, the conditions were 40 cycles at 94˚C for 15 s followed by 40 cycles at 60˚C for 1 min. For real-time PCRs were 40 cycles at 94˚C for 15 s followed by 40 cycles at 60˚C for 1 min. The relative mRNA expression levels were normalized to the levels of HPRT mRNA.

For some experiments, the ankles were used because they provide an easier assessment of reduced mobility by inflammation than do knee joints. Quantitative PCR analysis was performed using SYBR Green master mix, and the results of each sample were normalized to the results of the housekeeping gene. The relative mRNA expression levels were normalized to the levels of HPRT mRNA.
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 × 105 cells/well) and stimulated with human IL-6 (50 ng/ml; Toray Industries) plus human soluble IL-6R (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-kB-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-IFN-γ 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 on 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 with 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 (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).

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 GolgiPlugs (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, 106 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.

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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, 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). Thus, cells that responded to EGF family growth factors were defined as type 1 collagen+vimentin+ synovial fibroblasts and concluded to synthesize growth factors.

To investigate how each growth factor enhances the expression of chemokines and IL-6, we employed the cell line BC1, because we have found a significant enhancement effect on IL-6 and chemokine expressions after stimulations of IL-17 and IL-6 in this line

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, Tgfα, 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 × 10⁵ transducing units [TU], n = 6), Areg (1.9 × 10⁵ TU, n = 6), Btc (1.9 × 10⁵ TU, n = 6), Tgfα (1.9 × 10⁵ TU, n = 6), Fgf2 (1.9 × 10⁵ TU, n = 6), Plgf2 (1.9 × 10⁵ TU, n = 6), TNC (1.9 × 10⁵ TU, n = 6), a nontarget sequence (1.9 × 10⁵ TU, n = 6), or saline with neither IL-6 nor IL-17 (n = 6) on days 0, 1, 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 (PLGF) Ab (1 μg, n = 12), anti-TNC Ab (1 μg, n = 12), or 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-Plgf 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 (Fig. 2B–D). These results suggest that the pathways of Areg, BTC, TGF-α, or FGF2 are directly involved in the enhanced 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 (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 and D) mRNA expressions of IL-6 (C) and CCL20 (D) in BC1 cells 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 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). (E) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα in the presence or absence of Areg, BTC, TGF-α, or FGF2 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 mouse IL-6. Cell survival was evaluated based on mitochondrial activity. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test). (F) BC1 cells were stimulated with human IL-6 plus soluble IL-6Rα and mouse IL-17 in the presence or absence of Areg (A), BTC (B), TGF-α (T), or FGF2 (F) for 30 min and then investigated for the phosphorylation of Akt and p65. (G) 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

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**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 in the presence or absence of joint injections of anti-Ereg Ab (1 μg, n = 12), 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-PLGF2 Ab (1 μg, n = 12), anti-TNC Ab (1 μg, n = 12), or control IgG (1 μg, n = 12) on days 0, 1, 2, 4, and 6 followed by analysis of the expressions of Ereg, Areg, Btc, TGF-α, FGF2, Plgf2, and Tnc (D) and Ereg (E) in joint synovial tissues on day 7. (F–I) 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, Plgf2, 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 EAE mice were i.v. transferred into wild-type C57BL/6 mice in the presence or absence of anti–TGF-α Ab administration (i.p., days 0–5 after the pathogenic CD4+ T cell transfer). (C) Clinical EAE scores (n = 5 each) and (D) serum IL-6 concentrations in mice (n = 15). (E) Mononuclear cells 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
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