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frequent. A population-based study in this area indicated a U-shaped relationship between plasma HDL-C level and the incidence of ischemic electrocardiographic changes. In subjects with HDL-C <1.81 mmol l^{-1} (70 mg dl^{-1}), the incidence increased in proportion to the HDL-C levels. The frequency of the *IN14* CETP gene mutation was higher in patients with CHD than in control subjects. In subjects aged >80 years, the prevalence of both marked hyperalphalipoproteinemia and the *IN14* splicing defect was significantly lower than in the younger generation. Thus, a marked hyperalphalipoproteinemia caused by *IN14* CETP gene mutation does not represent a longevity syndrome, suggesting the importance of re-evaluation of the clinical significance and pathophysiology of a marked hyperalphalipoproteinemia.

The relationship between CETP gene mutations and CHD in Japanese American men in the Honolulu Heart Program cohort was evaluated. Men, most of whom had a *D442:G* mutation, showed an increased risk of CHD, but the atherogenic effect of CETP deficiency was observed in subjects with moderately increased HDL-C levels but not in subjects with a marked hyperalphalipoproteinemia. In the Framingham Heart Study, plasma CETP activity was measured in 1978 participants and on the average follow-up of 15 years, 320 participants experienced a first cardiovascular disease event (fatal or nonfatal coronary heart disease, cerebrovascular disease, peripheral vascular disease, or heart failure). In multivariable analyses, plasma CETP activity was related inversely to the incidence of cardiovascular disease events (hazard ratio for activity, at or above the median of 0.72; 95% CI, 0.57–0.90; P=0.004 [compared with below median]; hazard ratio per SD increment, 0.86; 95% CI, 0.76–0.97; P=0.01). These prospective data suggested that lower plasma CETP activity was associated with a greater cardiovascular disease risk. Similar data have been reported from the Ludwigshafen Risk and Cardiovascular Health (LURIC) study in Europe, in which low plasma CETP mass levels were associated with increased cardiovascular and all-cause mortality. In LURIC study, decreased cholesterol efflux capacity was shown in patients with low plasma CETP mass levels. Furthermore, the *post hoc* analyses of KAROLA study demonstrated a similar tendency in relations between low plasma CETP mass levels and cardiovascular and all-cause mortality. Thus, these studies challenge the rationale of pharmacological CETP inhibition.

Inhibiting CETP activity may raise HDL-C and decrease LDL-C levels. Thus, a number of CETP inhibitors have been developed. The initial clinical trial (ILLUMINATE Study) with a CETP inhibitor, torcetrapib, in combination with atorvastatin was prematurely terminated because of an increased cardiovascular event rate and mortality in torcetrapib-treated patients. RADIANCE1 and RADIANCE2 studies examining the effect of torcetrapib on carotid intima-media thickness in patients with familial hypercholesterolemia and mixed dyslipidemia, respectively, were also terminated. No beneficial effect of torcetrapib was observed on carotid intima-media thickness. ILLUSTRATE study also showed a small favorable effect for torcetrapib in the change in normalized atheroma volume, however there was no significant difference in the change in atheroma volume for the most diseased vessel segment, suggesting that torcetrapib may have no significant effect on the progression of coronary atherosclerosis. The lack of efficacy of torcetrapib may be related to the mechanism of action of this drug class or to molecule-specific adverse effects. Torcetrapib significantly increased blood pressure because of the increase in aldosterone levels.

Dalcetrapib is a CETP inhibitor which raises plasma HDL-C levels, but does not decrease plasma LDL-C. Dal-OUTCOMES study investigated the effect of dalcetrapib, at a dose of 600 mg daily, or placebo, in addition to the best available evidence-based care on the risk of recurrent cardiovascular events who had a recent acute coronary syndrome. The study was also terminated because dalcetrapib increased HDL-C levels, but did not reduce the risk of recurrent cardiovascular events. Studies on other CETP inhibitors such as anacetrapib and evacetrapib are still going on. However, a recent evidence suggests that CETP inhibitors form a complex between themselves, CETP and HDL particles, which might interfere with many physiological functions of HDL. Therefore, CETP inhibition is not a good target of HDL-modifying therapy.

In contrast to CETP inhibitors, an enhancement of RCT may be a promising strategy. A lipid-lowering drug, probucol, has a long history of clinical application with established efficacy and safety profiles. It is a potent antioxidant drug that has been in clinical use during the past few decades for the treatment and prevention of cardiovascular diseases. As mentioned above, probucol is a unique drug because it reduces plasma HDL-C levels, but attenuates xanthomas. Its mechanism of pharmacologic actions at the molecular level has recently been elucidated. HDL-C reduction by probucol is based upon the increase in plasma CETP activity and hepatic SR-BI expression, and may not be a 'side effect' but it most likely might reflect an acceleration. Probucol inhibits the oxidation of LDL and enhances the cholesterol efflux capacity and anti-oxidative function of HDL particles because it transforms HDL particles to cholesterol-poor small ones and it increases the expression of PON-1 on HDL. The molecular bases of the various anti-atherogenic effects of probucol are illustrated in Figure 3. Probucol could be reconsidered as an option at least in case statins, which are known to be effective for lowering LDL-C levels and CHD risk, are not effective. A marked CHD risk reduction has recently been reported in long-term probucol treatment of patients with heterozygous familial hypercholesterolemia FH as well as those after coronary revascularization. Therefore, there is more than enough reason to believe that this old drug has much more to offer than known so far.

Hepatic triglyceride lipase (HL) deficiency

HL plays a role in the conversion of IDL into LDL by its TG lipase activity. HL also has the function for the remodeling of large, TG-rich HDL particles into smaller ones by hydrolyzing TG of HDL. HL also enhances the hepatic uptake of HDL lipids. Several mutations have been reported in the human *HL* gene. Human HL deficiency is characterized by increased IDL-cholesterol levels as well as large and TG-rich HDL particles. Some patients with HL deficiency were reported to have premature atherosclerosis.

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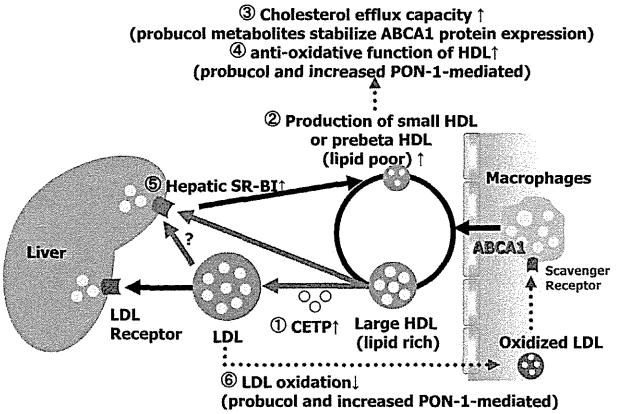

Figure 3 Molecular mechanisms for anti-atherogenic effects of probucol.

Familial hyperalphalipoproteinemia with premature corneal opacity (combined deficiency of CETP and HL activity)

Several patients with a combined reduction in CETP and HL activity were reported to present with corneal arcus and suffer from CHD. The impact of the combined reduction on atherosclerosis appears to be stronger than that of CETP deficiency alone. One of the possible mechanisms is that both CETP and HL play important roles in the remodeling of HDL particles from large to small particles, which are relatively more active for cholesterol efflux. The combined reduction of CETP and HL leads to the marked elevation of HDL-C, with the appearance of very large HDL particles, which are not active for cholesterol efflux.

Familial hyperalphalipoproteinemia with genetic abnormalities in SR-BI gene

Hepatic SR-BI takes up the cholesteryl ester from HDL particles. Adenovirus-mediated hepatic overexpression of SR-BI in mice resulted in virtual disappearance of plasma HDL and a substantial increase in biliary cholesterol. In contrast, the deletion of hepatic SR-BI increased plasma HDL-C, but enhanced the development and progression of atherosclerosis in mice.

From subjects with elevated HDL-C levels, a family with a new missense mutation (P297S) in the *SR-BI* gene has been identified. Cholesterol uptake from HDL by primary murine hepatocytes that expressed mutant SR-BI was reduced to half of that of hepatocytes expressing wild-type SR-BI. The P297S carriers showed increased HDL-C levels and a reduced capacity for cholesterol efflux from macrophages. However, the carotid artery intima-media thickness was similar in carriers and in family noncarriers. Platelets from carriers had increased unesterified cholesterol content and impaired function. In carriers, adrenal steroidogenesis was attenuated. Recently, two point mutations in human SR-BI gene, S112F or T175A, were also identified in subjects with high HDL-C levels.

Familial hyperalphalipoproteinemia with genetic abnormalities in EL gene

EL hydrolyzes the phospholipids of HDL. The gene of human EL is *LIPG*. Several genetic mutations or polymorphisms in the *LIPG* gene were reported. They show an increase in plasma HDL-C levels. However, recent a mendelian randomization study revealed that carriers of the *LIPG* Asn396Ser allele had higher HDL-C levels. However, the 396Ser allele was not associated with reduced risk of myocardial infarction. These data challenge the concept that raising of plasma HDL-C by inhibition of EL to reduce the risk of myocardial infarction.

Familial hyperalphalipoproteinemia with increased production of Apo A-I

A family with a marked hyperalphalipoproteinemia was reported to have an overproduction of apo A-I. The primary cause(s) is not known. We reported a hyperalphalipoproteinemic family with predominant increase in HDL_3. The cause of this hyperalphalipoproteinemia is speculated to be an increased production of apo A-I.

Familial hyperalphalipoproteinemia with reduced uptake of HDL by lymphocytes

A case of a marked hyperalphalipoproteinemia due to a reduced uptake of HDL by lymphocytes was reported. The molecular mechanisms for the reduction of HDL uptake by lymphocytes are unknown.

Secondary High HDL Syndrome

Chronic heavy alcohol consumption

Chronic heavy alcohol consumption is known to increase plasma HDL-C levels. Some enzymes and transfer proteins, such as CETP and HL, are altered in chronic heavy alcohol drinkers. CETP activity is reduced in these drinkers, but is normalized after cessation of

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alcohol. The association between alcohol intake and mortality is U-shaped, suggesting that the beneficial effect of alcohol intake is only observed in mild to moderate drinkers.

Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is a primary cholestatic liver disease, but its primary defect is unknown. In the end-stage of PBC, patients have very low HDL-C with the appearance of Lp-X, similar to other obstructive or cholestatic liver diseases. In the early stage of PBC, patients often demonstrate high HDL syndrome. In some cases, plasma HDL-C is markedly increased to the same extent as that in patients with CETP deficiency. In contrast to CETP deficiency, both activities and protein mass of CETP are markedly increased, whereas HL is reduced in patients with PBC.

Inhibitors of CETP in plasma

Defect in cholesteryl ester transport in serum of patients with uremia receiving maintenance hemodialysis was reported, suggesting an increased inhibitor activity against CETP in these patients.

Other factors or disease states accompanied by hyperalphalipoproteinemia

Aerobic exercise increases plasma HDL-C levels. Thus, hyperalphalipoproteinemia is sometimes observed in subjects who are continuously doing aerobic exercises. Patients with multiple symmetric lipomatosis, chronic obstructive pulmonary disease (COPD) and hypothyroidism are sometimes accompanied by hyperalphalipoproteinemia, but its mechanism is unknown except that HL activity is reduced in hypothyroidism.

Drugs

Some drugs, such as insulin, glucocorticoids, estrogen derivatives, fibrates, HMG-CoA reductase inhibitors (statins), nicotinic acids and their derivatives, intestinal cholesterol transporter inhibitor (ezetimibe), and cyclosporin were reported to increase plasma HDL-C levels. Prospective studies using a fibrate, gemfibrozil, demonstrated that increases in HDL-C during treatment were correlated with the prevention of cardiac events.

Conclusion

Various factors are involved in the etiology of low HDL syndrome and high HDL syndrome. A marked hyperalphalipoproteiemia caused by genetic CETP deficiency may not be protected from atherosclerotic cardiovascular diseases, therefore strategies to raise plasma HDL-C levels by CETP inhibitors have been challenged without a success. The enhancement of RCT by drugs such as probucol may have a greater potential as an anti-atherosclerotic treatment despite reduction of plasma HDL-C. The pleiotropic functions of HDL and efficiency of HDL-mediated RCT should be tested when we develop an HDL-targeted drug therapy for prevention of atherosclerosis.

Acknowledgments

This work was supported by a grant from the Japan Health and Labor Sciences Research Grant for Research on Rare and Intractable Disease to S. Y. This work was also supported by a grant-in-aid (No. 24390233 and 2667045) to S. Y. from the Ministry of Education, Science, and Culture of Japan.

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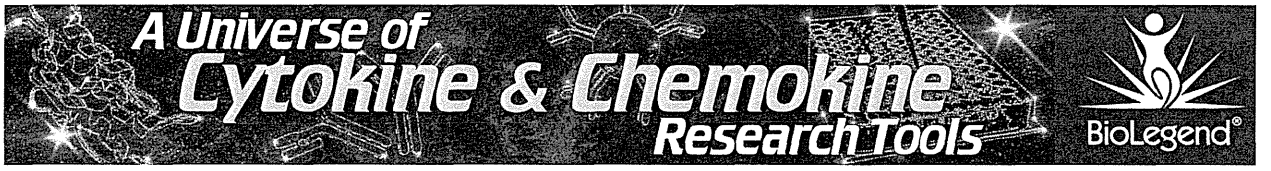

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J Immunol 2015; 194:1039-1046; Prepublished online 2 January 2015;
doi: 10.4049/jimmunol.1400562
http://www.jimmunol.org/content/194/3/1039

Supplementary Material **http://www.jimmunol.org/content/suppl/2015/01/02/jimmunol.1400562.DCSupplemental.html**

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Temporal Expression of Growth Factors Triggered by Epiregulin Regulates Inflammation Development

Masaya Harada,*,†,‡,§,[1] Daisuke Kamimura,*,†,‡,§,[1] Yasunobu Arima,*,†,‡,§ Hitoshi Kohsaka,¶ Yuji Nakatsuji,‖ Makoto Nishida,# Toru Atsumi,*,†,‡,§ Jie Meng,*,†,‡,§ Hidenori Bando,*,†,‡,§ Rajeev Singh,* Lavannya Sabharwal,*,†,‡,§ Jing-Jing Jiang,*,†,‡,§ Noriko 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 tenascin 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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Received for publication March 18, 2014. Accepted for publication November 25, 2014.

This work was supported by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (KAKENHI; to Y.A., M.H., M.M., and T.H.) and the Japan Science and Technology Agency–Core Research for Engineering, Science, and Technology program (to T.H. and M.M.), the Takeda Research Foundation (to M.M. and Y.A.), the Mochida Memorial Foundation (to Y.A.), the Japan Intractable Diseases Research Foundation (to Y.A.), the Uehara Foundation (to M.M.), the Naito Foundation (to M.M.), the Waksman Foundation of Japan (to M.M.), the Tokyo Biochemical Research Foundation (to M.M.), the Osaka Cancer Research Foundation (to M.M.), and the Osaka Foundation for the Promotion of Clinical Immunology (to M.M.).

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The online version of this article contains supplemental material.

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, tenascin C.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1400562

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 tenascin C (TNC) in patients were measured by a Milliplex kit (Merck, Tokyo, Japan) followed by analysis with a multiplex analysis device (Bio-Rad Laboratories, Tokyo, Japan).

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 protocols of the Osaka University Medical School. All animal experiments were performed following 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 (Ser^{536}, 93H1), anti–phospho-Akt (Ser^{473}, 193H12), 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: allophycocyanin-conjugated anti–IFN-γ (eBioscience, San Diego, CA) and control IgG1κ (eBioscience); FITC-conjugated anti-CD8 (eBioscience), anti-CD11b (Beckman Coulter, Brea, CA), anti-CD11c (eBioscience), anti-CD19 (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, MOG_{33-55}, pertussis toxin, IFA, 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-6Rα 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 (Toray 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/revers): mouse HPRT primers, 5′-GATTAGCGATGATGAACCAGGTT-3′ and 5′-CCTCCCATCTCCTTCATGACA-3′; mouse IL-6 primers, 5′-GAGGATACCACTCCCAACAGACC-3′ and 5′-AAGTGCATCATCGTTGTTCATACA-3′; mouse CCL20 primers, 5′-CGACTGTTGCCTCTCGTACA-3′ and 5′-GAGGAGGTTCACAGCCCTTT-3′; mouse epiregulin primers, 5′-CTGCCTCTTGGGTCTTGACG-3′ and 5′-GCGGTACAGTTATCCTCGGATTC-3′; mouse Areg primers, 5′-GCAGATACATCGAGAACCTGG-3′ and 5′-CTGCAATCTTGGATAGGTCCTTG-3′; mouse BTC primers, 5′-AATTCTCCACTGTGTGGTAGCA-3′ and 5′-GGTTTTCACTTTCTGTCTAGGGG-3′; mouse TGF-α primers, 5′-CACTCTGGGTACGTGGGTG-3′ and 5′-CACAGGTGATAATGAGGACAGC-3′; mouse FGF2 primers, 5′-GAGTTGTGTCTATCAAGGGAGTG-3′ and 5′-CCGTCCATCTTCCTTCATAGC-3′; mouse PLGF2 primers, 5′-TCTGCTGGGAACAACTCAACA-3′ and 5′-GTGAGACACCTCATCAGGGTAT-3′; mouse TNC primers, 5′-CACAACCCGTGAGTACCAGC-3′ and 5′-AGAGGGTATGCTATAAGCCAGAA-3′; mouse E-cadherin primers, 5′-CCAATCCTGATGAAATTGGAAACT-3′ and 5′-CGTAATCGAACACCAACAGAGAGT-3′; and mouse β-actin primers, 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′. The conditions for real-time PCRs were 40 cycles at 94°C for 15 s followed by 40 cycles at 60°C for 60 s. 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 restricted mobility by inflammation than do knee joints. Quantitative PCR analysis was performed using knee tissues, because a larger amount of RNA can be obtained and there is no need to pool samples from several mice, which reduces the number of animals used. We confirmed that the quantitative PCR results were equivalent between ankle and knee samples.

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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-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-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 MOG_{35-55} 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 using anti-CD4 microbeads (Miltenyi Biotec, Tokyo, Japan). The resulting CD4$^+$ T cell–enriched population (4×10^6 cells) was cocultured with rIL-23 (10 ng/ml; R&D Systems) in the presence of MOG peptide–pulsed irradiated splenocytes (1×10^7 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.

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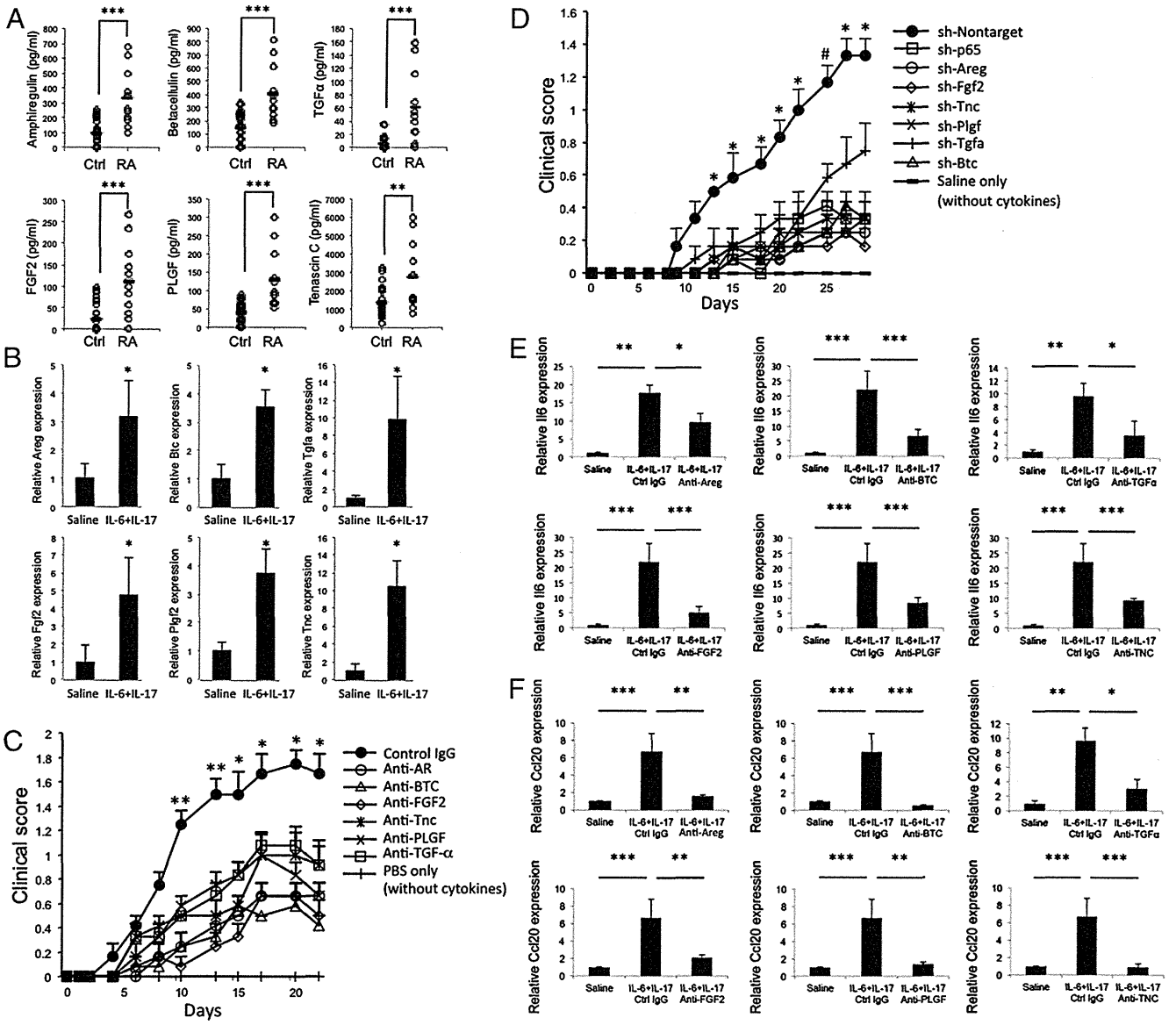

FIGURE 1. Areg, BTC, TGF-α, FGF2, PLGF, and TNC are critical for the development of cytokine-induced arthritis. (**A**) Serum concentrations of Areg, BTC, TGF-α, FGF2, PLGF, and TNC in patients suffering from RA (n = 11) compared with healthy age- and sex-matched subjects (n = 26). (**B**) IL-17A (0.2 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) (1.9×10^5 transducing units [TU], n = 6), Areg (1.9×10^5 TU, n = 6), Btc (1.9×10^5 TU, n = 6), Tgfa (1.9×10^5 TU, n = 6), Fgf2 (1.9×10^5 TU, n = 6), Plgf2 (1.9×10^5 TU, n = 6), TNC (1.9×10^5 TU, n = 6), a nontarget sequence (1.9×10^5 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-Plgf2 (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).

Areg, BTC, TGF-α, and FGF2 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

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(4, 5, 10, 16, 17). 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-

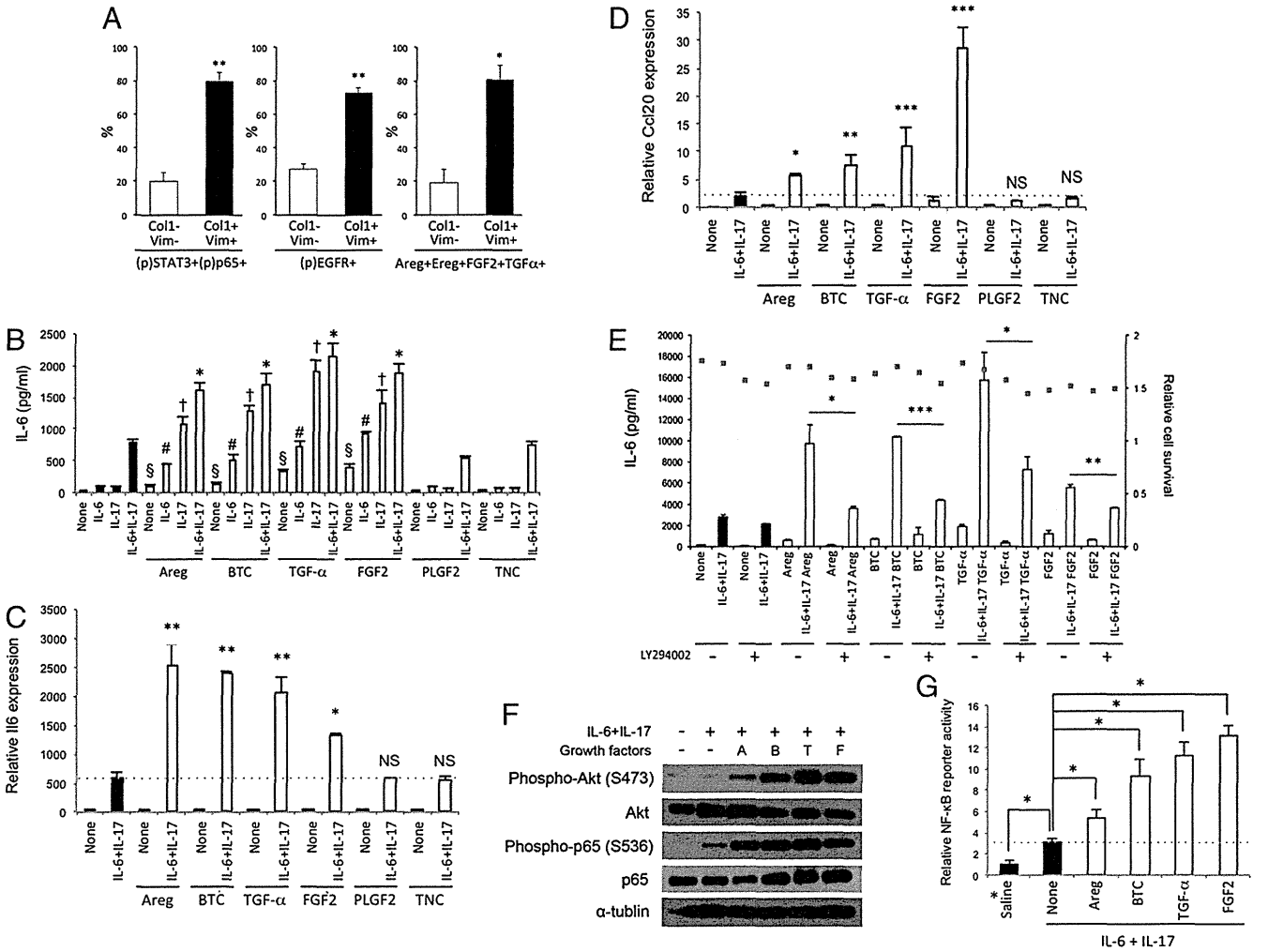

FIGURE 2. Areg, BTC, TGF-α, and FGF2 enhance the expressions of IL-6 and chemokines via the PI3K/NF-κB axis. (**A**) IL-6 (1 μg) and IL-17 (1 μg) were injected into the left ankle joints of F759 mice on days 0, 1, and 2. Immunohistochemistry of the left ankle joints was performed by using Abs against 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. $^{\S}p < 0.05$, $*p < 0.01$, $^{\#,\dagger}p < 0.001$ (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

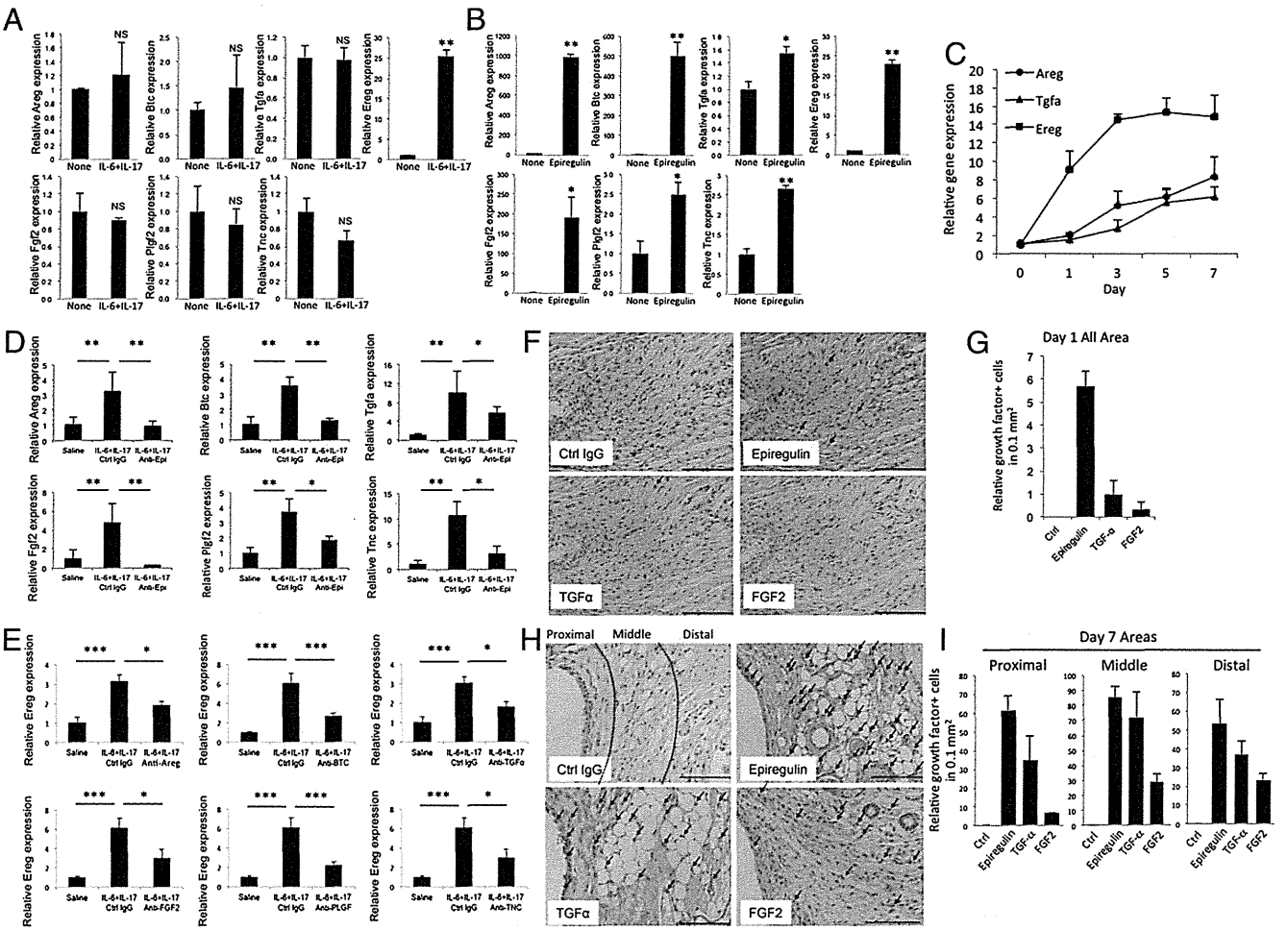

FIGURE 3. Presence of epiregulin-triggered temporal expressions in affected tissues of cytokine-induced arthritis. (**A**) mRNA expressions of Areg, Btc, Tgfa, epiregulin (Ereg), Fgf2, Plgf2, and Tnc in BC1 cells in the presence or absence of stimulation with IL-17 and IL-6 were evaluated 3 h later using 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 mm^2 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). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

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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.

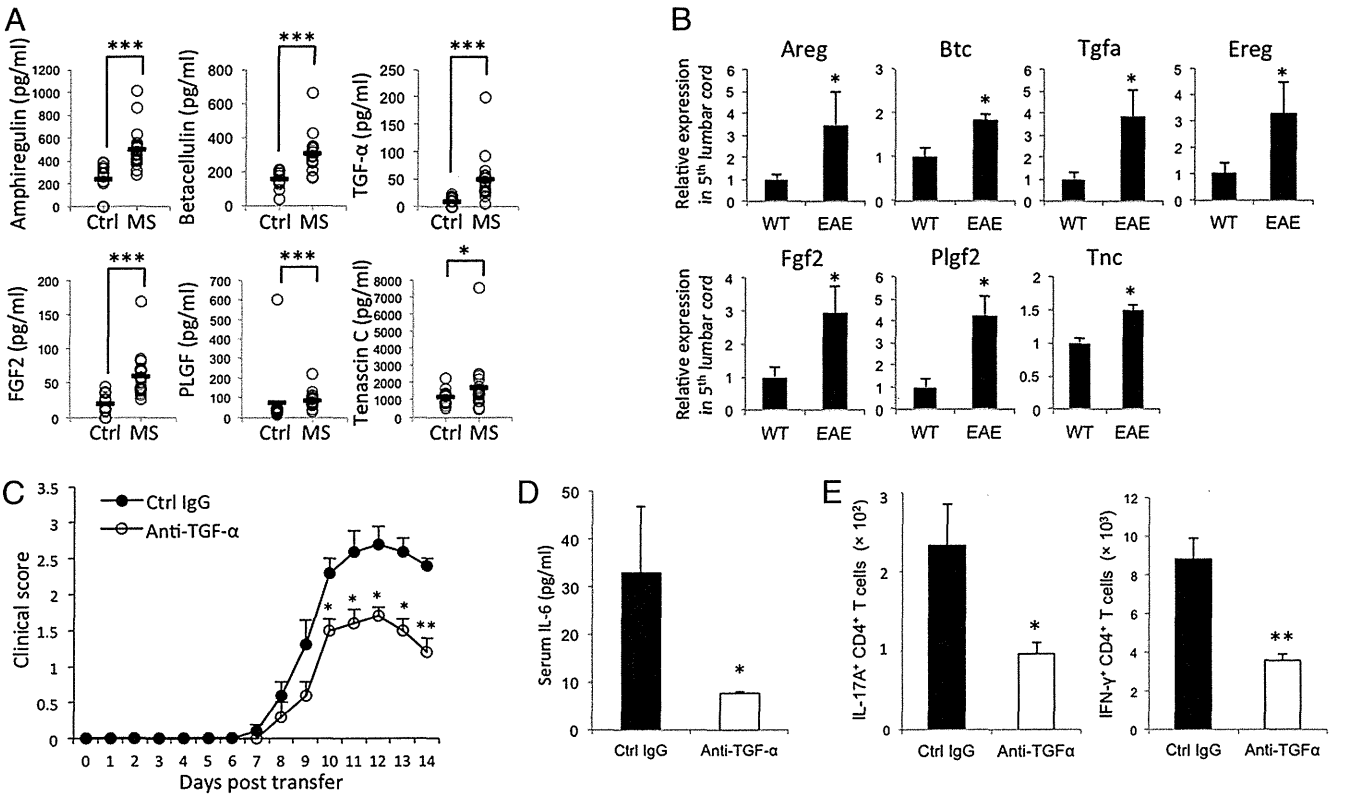

FIGURE 4. Growth factors are critical for the development of an MS model, EAE. (**A**) Serum concentrations of Areg, BTC, TGF-α, FGF2, PLGF, and TNC in patients suffering from MS ($n = 21$) compared with healthy age- and sex-matched subjects ($n = 15$). (**B**) mRNA expressions of epiregulin (Ereg), 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 $CD4^+IL\text{-}17^+$ and $CD4^+IFN\text{-}\gamma^+$ 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.

Acknowledgments

We thank Dr. Peter Karagiannis (RIKEN Quantitative Biology Center, Osaka, Japan) for carefully reading the manuscript. We also thank Dr. Seiritsu Ogura (Hosei University, Tokyo, Japan) for providing critical statistical advice.

Disclosures

The authors have no financial conflicts of interest.

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