Amelioration of experimental arthritis by the intra-articular injection of an epidermal growth factor receptor tyrosine kinase inhibitor

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Abstract

Objective

Selectively targeting signalling pathways represents a promising pharmacological approach in rheumatoid arthritis (RA). Abundant levels of epidermal growth factor receptor (EGFR) are expressed in the synovial lining layers, and the anti-arthritis effect of erlotinib and lapatinib, small-molecule EGFR tyrosine kinase inhibitors (TKIs), has been demonstrated through the systemic administration on experimental arthritis models. Nevertheless, their therapeutic responses by the intra-articular (i.a.) route remain to be explored in rheumatoid joint.

Methods

The administration of an EGFR TKI (a gefitinib analogue) was explored in two in vivo models of collagen-induced arthritis (CIA) and in vitro experiments by using synovial fibroblasts (SF) from RA patients and CIA rats.

Results

There was a significant reduction of arthritis scores in CIA mice receiving the daily intraperitoneal injection. After the onset of arthritis in CIA rats, ankle joints receiving a single i.a. injection had significant lower articular indexes with reduced synovial inflammation, pannus formation and erosion on cartilage and bone as well as total histological scores by histopathological analyses. In CIASF or RASF, upon in vitro human EGF stimulation, there was a dose-dependent increase in cell proliferation and Akt activation with suppressed responses under the EGFR TKI treatment.

Conclusion

These findings demonstrate the effect of i.a. injection of an EGFR TKI on amelioration of rheumatoid joint through the suppression of synovial inflammation, pannus formation and erosion on cartilage and bone in experimental arthritis, implicating targeting the i.a. EGFR signalling transduction as a pharmacological strategy.

Key words

epidermal growth factor receptor, intra-articular injection, rheumatoid arthritis, signalling transduction, tyrosine kinase inhibitor

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Introduction

Several intracellular signalling pathways have been explored in rheumatoid joint like Janus kinase (JAK) and phosphatidylinositol 3-kinase pathways, and significant responses in the trials of a JAK 1/3 inhibitor have allowed FDA to approve the first targeted agent in rheumatoid arthritis (RA) therapy, indicating that selectively targeting signalling transduction tracks represents a promising pharmacological strategy (1, 2). Epidermal growth factor receptor (EGFR), a well-known transmembrane tyrosine kinase growth factor receptor family, is regulated by different activating ligands with EGF as the prototype (3). In RA, EGF is constitutively produced by RA synovial fibroblasts (SF), and abundant levels of EGFR are expressed in the synovial lining layers (4). Interestingly, a recent study reveals that IL-1β can induce the release of EGF from RASF, and the prolong remission of arthritis activities has been observed in the concurrent RA and oncology disease status after completing the treatment with cetuximab, a monoclonal antibody against EGFR (5, 6). The role of EGFR is further supported by the suppression effect of intravenous injection with adenoviral vectors carrying the EGFRs inhibitor in an arthritis model, implicating EGFR antagonism as a therapeutic approach (7). Small-molecule EGFR tyrosine kinase inhibitors (TKIs) including gefitinib, erlotinib and lapatinib, can compete reversibly with ATP to bind to the tyrosine kinase domain with further inhibition of the downstream signalling pathways (3). Indeed, by the systemic administration of erlotinib or lapatinib through oral gavage, two studies have shown the anti-arthritis effect with reduction of synovial inflammation and erosion on cartilage and bone in experimental models; however, their therapeutic responses by the intra-articular (*i.a.*) route remain to be explored (4, 8).

In this study, the administration of a small-molecule EGFR TKI (a gefitinib analogue) was performed in two *in vivo* arthritis models and *in vitro* experiments by using SF from RA patients and CIA rats. We observed the therapeutic effect on amelioration of arthritis by the *i.a.* injection of an EGFR TKI, and further demonstrated inhibition of synovial inflammation, pannus formation and erosion on cartilage and bone by histopathological analyses.

Materials and methods

Synovial samples from RA patients

Synovial tissues were obtained with the approval from the Institutional Review Board of the National Cheng Kung University Hospital.

Induction of CIA in mice and rats and isolation of SF from synovial tissues

Male DBA/1 (J) mice (8 weeks of ages) from the Jackson Laboratory and male Sprague-Dawley rats (8 weeks of age) from the Laboratory Animal Centre in National Cheng Kung University Medical College, were housed under specific pathogen-free condition. To induce CIA in mice, they received the intradermal (*i.d.*) immunisation with bovine type II collagen (Elastin Products) in Freund's complete adjuvant (Chondrex) on day 0 and a booster on day 21, as described previously (9). To induce CIA in rats, they were immunised by the *i.d.* injection with bovine type II collagen in Freund's complete adjuvant on days 0 and 7, as described previously (10). The synovial tissues were treated with collagenase and incubated overnight, and adherent SF were cultured continuously until confluence with the usage of lines between 4th and 7th passages in this study, as described previously (10).

Therapeutic protocols and evaluation in arthritis models

The small-molecule EGFR TKI (AZ11860334), a potent and selective inhibitor with pharmacological and physiochemical characteristics similar to gefitinib (11), was kindly provided by AstraZeneca UK limited, and we first tested its anti-arthritis effects on CIA mice. They received daily intraperitoneal (*i.p.*) injection starting from day 30 with PBS as the control (5 mice per group), and were evaluated for clinical signs from 0 to 3 in each paw with a maximum score of 12 in a mouse (12). For the *i.a.* therapeutic protocol in CIA rats, the right ankle joints received this

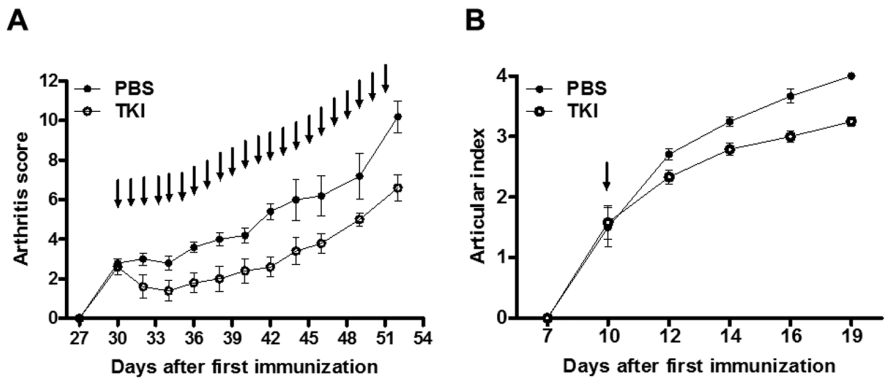

Fig. 1. Amelioration of arthritis in experimental arthritis models.
A. CIA mice received the daily *i.p.* injection of 10 μM compound with the PBS injection as control counterparts (5 mice per group) from day 30, and were evaluated for arthritis scores (p=0.0116). Each value represents the mean ± SEM, and the arrows indicate time of injection.
B. Right ankle joints of CIA rats received an i.a. injection of 5 μM compound with the PBS injection into left ankles as control joints (12 joints per group) on day 10, and were evaluated for articular indexes (p=0.0049). Each value represents the mean ± SEM, and the arrow indicates time of injection. All of the results in Fig. 1 are representative of at least two independent experiments with similar results.

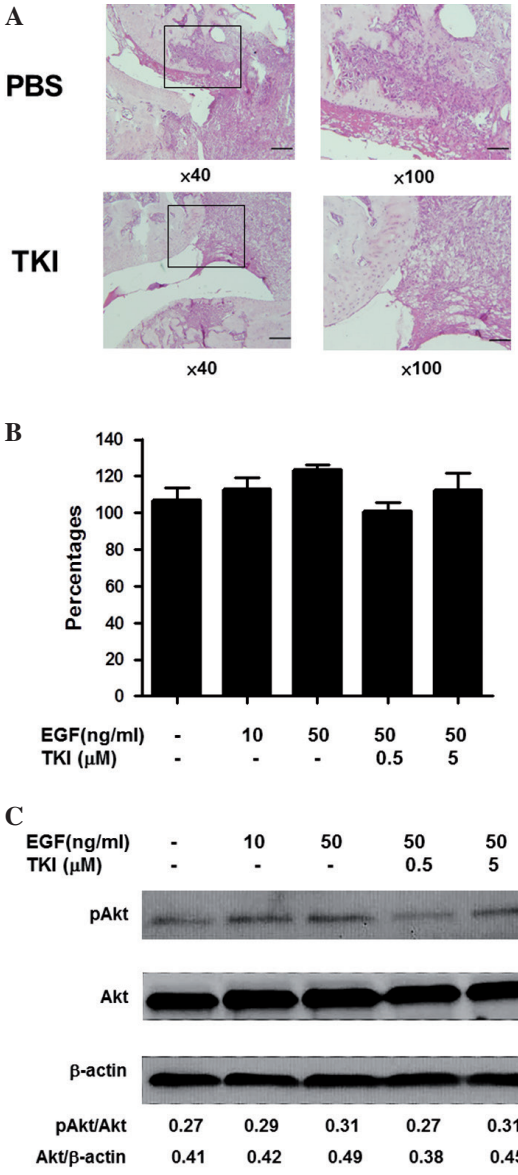

Fig. 2. Analysis of CIA rats receiving the *i.a.* EGFR TKI injection with histopathological examinations and in vitro SF experiments.
A. Representative histopathological images of an ankle joint receiving 5 μM compound treatment (lower) with less synovial inflammation, pannus formation, cartilage erosion and bone erosion as compared with a control PBS-injected joint (upper) by H&E staining. Scare bars represent 500 μm in ×40 magnifications (left) and 200 μm in ×100 magnifications (right).
B. A dose-dependent increase in cell proliferation of CIASF (n=3) by using colorimetric WST-8 assay in the presence of human EGF and with an inhibition by the compound at 0.5 μM (p=0.0206).
C. A representative immunoblot analysis with a dose-dependent increase in Akt activation by the human EGF stimulation and an inhibition by EGFR TKI at 0.5 μM in CIASF. All of the results in Fig. 2B and 2C are representative of three independent experiments with similar results.

compound with the injection of PBS into left ankles as control joints on day 10 (12 joints per group), and the articular index for scoring was from 0 to 4, as described previously (10). Ankle joints were resected upon sacrifice on day 19, and hematoxylin and eosin (H&E)-stained joint sections received histopathological evaluation based on synovial inflammation, pannus formation, cartilage erosion and bone erosion with a scale of 0 to 3 for each item (0 = absent, 1 = mild, 2 = moderate, 3 = severe) and a total score of 12. There were one representative section taken from each joint and 5 joints per treatment group.

Cell proliferation assay

RASF or CIASF (5×10^3/well) were cultured in 96-well flat bottomed microplates for 72 hr in the presence of 10 or 50 ng/ml recombinant human EGF (R&D) and 0.5 or 5 μM EGFR TKI. Cell proliferation of RASF or CIASF was measured by pulsing culture cells with ^{3}H thymidine or colorimetric WST-8 assay, as described previously (13).

Immunoblot analysis

Cell lysates of CIASF or RASF were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Amersham), incubated with anti-phosphorylated Akt (Ser473) (Cell signalling), anti-Akt (Cell signalling) or anti-β-actin antibody (Sigma-Aldrich), followed by a horseradish peroxidase-labelled secondary antibody (Jackson ImmunoResearch), then developed with the ECL plus system (Amersham), and analysed by a Biospectrum imaging system (UVP) for chemiluminescence detection with the signal intensity quantitated by the Image J software (NIH), as described previously (10).

Statistical analysis

Data in this study were presented as mean ± SEM. Differences in arthritis scores or articular indexes were compared by repeated-measures analysis of variance, and values of experimental data between different groups were assessed with Student's t-test. P-values less than 0.05 were considered significant in this study.

Results

Amelioration of CIA by the i.a. administration of EGFR TKI

At first, the therapeutic effect on rheumatoid joint was tested in CIA mice by using the systemic administration through an *i.p.* route. Significant reduction of arthritis scores was demonstrated in CIA mice receiving 10 μm daily injection as compared with control mice (Fig. 1A, p=0.0116). After the confirmation of anti-arthritis efficacy, we examined the therapeutic effect on CIA rats through the *i.a.* administration route. CIA joints receiving a single *i.a.* injection on day 10 had significant lower articular indexes as compared with control counterparts (Fig. 1B, p=0.0049).

Further histopathological characterisation on ankle joints of CIA rats revealed that the treatment group in comparison with the control group had less synovial inflammation, pannus formation, cartilage erosion and bone erosion with significantly reduced individual score regarding synovial inflammation, pannus formation and bone erosion as well as total histological scores (Fig. 2A; Table I).

A dose-dependent enhanced proliferation of CIASF in the presence of human EGF was observed, and the addition of 0.5 μM EGFR TKI could significantly inhibit the response (Fig. 2B, p=0.0206). The effect of EGFR TKI on human EGF-mediated Akt activation was analysed in the presence of this compound at different concentrations. There was a dose-dependent increase in pAkt/Akt and pAkt/β-actin ratios under the human EGF stimulation, suppressed by the addition of 0.5 μM EGFR TKI (Fig. 2c).

Analyses of cell proliferation and Akt activation in SF from RA patients

Finally, we analysed cell proliferation and Akt activation in RASF. There was a dose-dependent enhanced cell proliferation in the presence of human EGF, and the addition of 0.5 μM EGFR TKI had a maximum inhibition on the response (Fig. 3A, p=0.0135). Similar to the findings by using the CIASF, a dose-dependently increased Akt activation with up-regulated pAkt/Akt and pAkt/β-actin ratios were demonstrated with the stimulation of human EGF, suppressed by the compound at the concentration of 0.5 μM (Fig. 3B).

In addition, we further tested the ability of TKI to block endogenous EGFR activation by examining the cell proliferation and Akt activation in RASF at the concentration of 0.5 μM alone without the exogenous EGF. Indeed, there were decreases in these responses in the presence of this compound as compared with the control without the addition of EGFR TKI (a reduction of 17% cell proliferation and 12% pAkt/Akt ratio in a representative experiment), in agreement with the previous findings that EGF is constitutively produced by RASF, further enhancing their proliferation in an autocrine fashion (4, 14).

Table I. Histopathlogical comparison of CIA joints receiving different treatments.

Histological characters	PBS control	EGFR TKI	p-value
Synovial inflammation	2.6 ± 0.3	1.4 ± 0.3	0.0085
Pannus formation	2.8 ± 0.2	1.8 ± 0.4	0.0462
Cartilage erosion	2.2 ± 0.2	1.6 ± 0.3	0.0943
Bone erosion	2.2 ± 0.2	1.4 ± 0.3	0.0353
Total histological score	9.8 ± 0.7	6.2 ± 0.7	0.0066

Data representing mean ± SEM in each histological item of different treatment group.

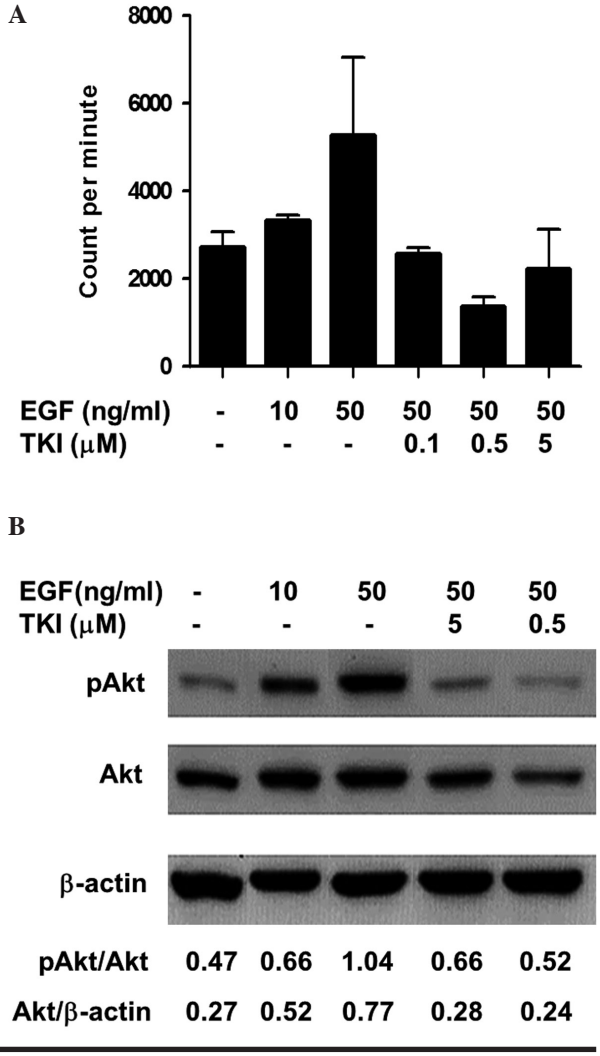

Fig. 3. Inhibition of cell proliferation and Akt phosphorylation by EGFR TKI in RASF. **A**. A dose-dependent increase in cell proliferation of RASF (n=3) by pulsing the cultured cells with ^{3}H thymidine in the presence of human EGF and an inhibition by EGFR TKI at 0.5 μM (p=0.0135). **B**. A representative immunoblot analysis with a dose-dependent increase in Akt activation by the human EGF stimulation and an inhibition by EGFR TKI at 0.5 μM. All of the results in Fig. 3 are representative of three independent experiments with similar results.

Discussion

Systemic administration of EGFR TKI is known to be associated with common adverse effects including skin rash and diarrhoea, and uncommon but fatal ones like pneumonitis (3). The *i.a.* in-

jection, a method bypassing the inherited side effects associated with the systemic administration, can provide a better bioavailability, fewer extra-articular adverse events and reduced total drug cost (15). Although the *i.a.* injection of TNF-α blockade is not recommended to treat refractory autoimmune arthritis, interestingly, one single administration of etanercept into a monoarticular rheumatoid joint achieves clinical improvement lasting up to 24 weeks (16, 17). Indeed, the use of *i.a.* route for EGFR TKI administration in CIA joints significantly reduced articular indexes and total histological scores, and such a proof-of-concept approach in animal experiments might further lead to the clinical validation of therapeutic effect. Nevertheless, regarding a persistent lifelong disease course with fluctuating activity in most RA patients, suitably modified injection schedules or the introduction of drug delivery system should be considered to evaluate the effect of *i.a.* administration in clinical studies (15, 18).

Although there is an extensive application of TNF-α blockades in rheumatoid joint, a significant number of patients fail to respond to such therapies, and to combine TNF-α inhibition with other biologics results in the impairment of host defense without enhancement of clinical efficacy (19). Interestingly, the combined systemic administration of EGFR bispecific ligand trap with low-dosage etanercept can abrogate arthritis in a mouse model, suggesting that simultaneous targeting EGFR and TNF-α signalling activation pathways is beneficent in rheumatoid joint (20). Furthermore, a novel strategy targeting SF has been proposed in RA therapy, and such an approach can avoid an interference of host defense against infection, an inevitable side effect associated with the usage of biologics (21). In this study, there was significant amelioration of rheumatoid joint through targeting the EGFR signalling pathway in SF. Notably, in addition to SF, the effect of systemic administration of erlotinib on amelioration of CIA mice has been demonstrated by targeting other non-immune cells like endothelial cells and osteoclasts with the evidence of reduced neoangiogenesis and osteoclastogenesis in inflamed joints (4), and further effort is required to examine whether endothelial cells and osteoclasts are also the target cell types in the experimental setting with the *i.a.* injection of EGFR TKI in arthritis models. Taken together, in the clinical scenario of RA patients with persistent monoarthritis refractory to the systemic TNF-α inhibitor treatment, there might remain a therapeutic modality to use the *i.a.* administration of EGFR TKI.

In conclusion, these findings demonstrate the therapeutic effect by using the *i.a.* injection of EGFR TKI on amelioration of rheumatoid joint by suppression of synovial inflammation, pannus formation and erosion on cartilage and bone in experimental arthritis.

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