**Figure 4** Effects of methotrexate (MTX) and corticosteroids on P-glycoprotein (P-gp) expression in patients with highly active rheumatoid arthritis (RA). Flow cytometric analysis for P-gp expression on peripheral CD4+ and CD19+ lymphocytes in 61 patients with highly active RA and DAS28 of more than 5.1 points treated with or without MTX or corticosteroids. Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data are mean (SD). *p<0.05, **p<0.01, by one-way ANOVA.
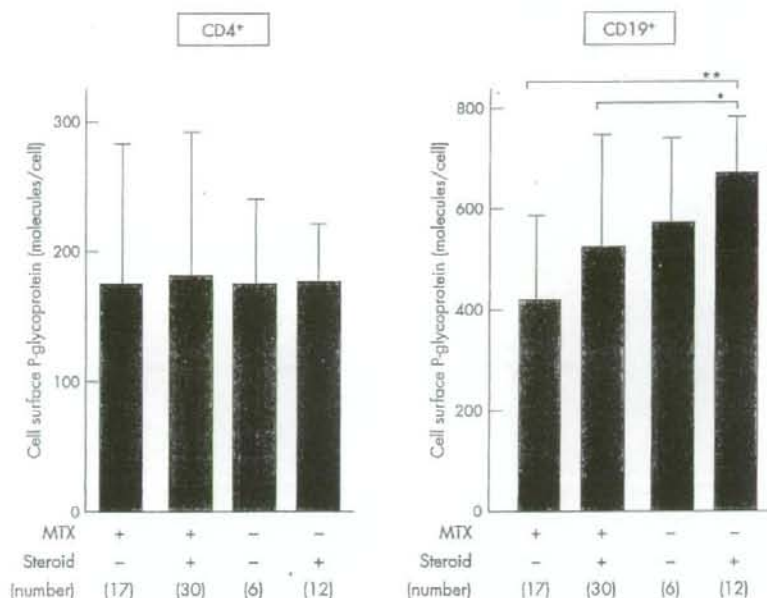
Table 3 Expression of P-glycoprotein on lymphocytes in rheumatoid arthritis (RA) patients with high disease activity

	DAS28-3	P-glycoprotein, molecules/cell CD4+ lymphocytes	CD19+ lymphocytes
Corticosteroids:			
Users (n = 42)	6.35 (0.72)	175.8 (94.3)	565.3 (204.1)
Non-users (n = 23)	6.22 (0.73)	169.7 (96.0)	459.6 (200.3)
p Value	NS	NS	<0.05
Methotrexate:			
Users (n = 47)	6.38 (0.73)	174.6 (106.9)	487.3 (216.6)
Non-users (n = 18)	6.21 (0.71)	171.3 (49.5)	633.9 (136.6)
p Value	NS	NS	<0.01

p Values are for comparison between groups with different characteristics in patients with high RA disease activity. Values are mean (SD).
DAS28-3, Disease Activity Score 28-3; NS, not significant.

and CD19+ lymphocytes of RA patients with high disease activity.

Other investigators and our group have reported that low cytoplasmic corticosteroid concentrations (caused by increased P-gp-mediated efflux of corticosteroid from lymphocytes) is one of the mechanisms of corticosteroid resistance in inflammatory bowel disease, asthma and systemic lupus erythaematosus.[11 37-39] Some DMARDs, eg, chloroquine, hydroxychloroquine, D-penicillamine and colchicines, are substrates of P-gp as well as corticosteroids.[8-10] Yudoh et al reported that P-gp expression on lymphocytes in RA patients might be also involved in resistance to bucillamine and sulfasalazine.[40] We noted that P-gp acts as a "hydrophobic vacuum cleaner", that is, P-gp captures those drugs that are substrates of P-gp when they pass through the cell membrane and then releases them outside the cell. Thus, when the number of P-gp molecules expressed on the lymphocyte cell surface increases, corticosteroids and some DMARDs that are substrates of P-gp, cannot reach the

cytoplasm, resulting in low response to treatment with these drugs. We demonstrated that the levels of intracellular dexamethasone in PBMCs from RA patients correlated closely with P-gp expression on lymphocytes. Our results imply that the high expression of P-gp on lymphocytes, which correlated

Table 4 Expression of P-glycoprotein on lymphocytes in rheumatoid arthritis patients with high disease activity, according to treatment

Methotrexate	Corticosteroids	n	DAS28-3
+	–	17	6.14 (0.67)
+	+	30	6.48 (0.72)
–	–	6	6.39 (0.85)
–	+	12	6.14 (0.69)

Values are mean (SD). There were no differences in DAS28-3 based on treatment regimen.
DAS28-3, Disease Activity Score 28-3.

385

Table 5 Infliximab infusion in 11 patients with refractory rheumatoid arthritis

Case	Age (years)	Disease duration (years)	Steinbrocker stage	Previously used DMARDs and PSL	DAS28-3
1	44	1	II	MTX	6.3
2	57	7	III	MTX, D-pen, SSZ	6.2
3	53	10	II	MTX, SSZ, PSL	5.2
4	40	14	III	MTX, PSL	5.8
5	76	10	II	MTX, SSZ	5.2
6	48	3	II	MTX, LFF, SSZ	6.0
7	60	15	IV	MTX, LFN	7.0
8	66	15	IV	MTX, Gold, PSL	7.6
9	51	17	IV	MTX, Gold, SSZ, PSL	7.4
10	55	9	IV	MTX, SSZ	7.6
11	60	7	IV	MTX, Gold, PSL	6.2

DAS28-3, Disease Activity Score 28-3; DMARD, disease-modifying antirheumatic drugs; D-pen, D-penicillamine; Gold, parenteral gold; LFN, leflunomide; MTX, methotrexate; PSL, prednisolone (or equivalent); SSZ, sulphasalazine.

with disease activity, might lead to active efflux of corticosteroids and P-gp substrate DMARDs to the cell exterior, resulting in the development of drug resistance and failure to control disease activity in RA patients with highly active disease.

Therefore, when one of the targets of overcoming treatment resistance is P-gp on lymphocytes, one of the good countermeasures could be administration of competitive inhibitors. Administration of substrates that have high affinity to P-gp, and inhibit P-gp mediated exclusion of other substrates, could overcome the multidrug resistance induced by P-gp expression. Tacrolimus is such a competitive inhibitor.[29 30] We demonstrated that levels of intracellular dexamethasone in PBMCs of highly active RA patients actually increased with tacrolimus treatment. Therefore, we suggest that tacrolimus could be used not only to inhibit NF-AT-dependent transcription of cytokines in lymphocytes, but also as a competitive inhibitor of P-gp, and propose that combination treatment with tacrolimus as a competitor of P-gp is a useful treatment for highly active refractory RA patients. Indeed, the efficacy of tacrolimus has been already reported in refractory RA patients.[28 41 42]

By contrast, MTX could also suppress P-gp expression on activated lymphocytes. Although MTX has been reported to efflux to the cell exterior through the ABCC subfamily of proteins, which are members of ATP-binding cassette transporters superfamily,[30 43] the mechanisms that regulate the ABCC subfamily on lymphocytes, relevance of the ABCC subfamily to RA clinical features and clinical relevance of the ABCC subfamily to drug resistance in RA, are not clear at present. Our results suggest that MTX might prolong the effects of combined DMARDs by preventing acquisition of drug resistance with restriction of P-gp expression in patients with highly active RA. Conversely, corticosteroid might enhance P-gp expression on activated lymphocytes, resulting in enhancement of multidrug resistance. In such cases, the dose of corticosteroids should be kept as low as possible.

We propose that overcoming treatment resistance might require reduction of P-gp levels on activated lymphocytes in patients with highly active RA. TNF plays a critical role in mediation of the pathogenic actions of inflammation and bone erosion in RA resulting in enhancement of RA disease activity, and thus is an important molecular target for directed biologic intervention.[14 17 18 44] It is noteworthy that infliximab successfully improved disease activity and reduced P-gp expression on

lymphocytes in 11 patients with refractory RA who had high levels of P-gp expression. In these patients, lymphocyte activation could not be suppressed when they were treated with MTX. Furthermore, we demonstrated the preferential expression of P-gp on activated subgroups (had high levels of CD69 expression) among CD4+ T cells.[12] In this report, infliximab infusion resulted in elimination of the P-gp high-expressing CD4+ lymphocytes. Therefore, we propose that infliximab inhibits activated lymphocytes resulting in reduction of P-gp expression. This is the first report that demonstrates the effect of infliximab on the reduction of P-gp and addressed the possible beneficial actions of infliximab. Recovery drug concentrations in lymphocytes associated with marked reduction of P-pg on lymphocytes by treatment with infliximab might result in overcoming treatment resistance in refractory RA. Translated clinically, these findings indicate that when a patient with RA fails to develop clinical remission with DMARDs and corticosteroids, overexpression of P-gp might be involved in treatment resistance. In such a case, a better option is administration of DMARDs and immunosuppressive agents that are not actively exteriorised from lymphocytes by P-gp, and, more properly, biological agents such as anti-TNF compounds, which are not affected by P-gp, should be initiated.

In conclusion, we have demonstrated in the present study that lymphocytes activated by various stimuli in RA patients with highly active disease apparently acquire MDR-1-mediated multidrug resistance against corticosteroids and probably some DMARDs, which are substrates of P-gp. Our results suggest that in patients with highly active RA who develop P-gp-mediated multidrug resistance, treatment with MTX is necessary first, and that it is not advised to supplement treatment with P-gp substrate DMARDs without P-gp inhibitors or increase the dose of corticosteroid only. Furthermore, our results suggest that the inhibition of P-gp by competitive inhibitors and the reduction of P-gp by biological agents could overcome drug resistance induced by P-pg expression on lymphocytes in refractory RA. Accordingly, we propose that measurement of P-gp expression level on peripheral blood lymphocytes is useful for the assessment of drug resistance and lymphocyte activation, and is a good tool for selection of DMARDs including P-gp competitors such as tacrolimus, and for application of biological agents in RA patients with highly active disease.

Figure 5 Effects of infliximab infusion on P-glycoprotein (P-gp) expression and intracellular dexamethasone levels in peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients with high disease activity. A. P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from 11 patients with highly active RA, whose Disease Activity Score (DAS)28 were more than 5.1 points, before (closed bars) and 14 days after (hatched bars) infliximab infusion. Data represent the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Values are mean and SD of 11 independent experiments. *$p < 0.05$, **$p < 0.01$ by paired t test. B. Typical P-gp expression on CD4+ and CD19+ peripheral blood lymphocytes from four patients with active RA (Cases 1, 2, 3 and 4 in table 5) before and 14 days after infliximab infusion. The dotted line represents the gate set to discriminate negative from positive stained cells as determined by control FITC-conjugated anti-mouse IgG Ab. C. Intracellular dexamethasone levels were evaluated by determining the cell to medium (C/M) ratio in PBMCs from the same patients. The C/M ratio was evaluated before (closed bars) and 14 days after (dotted circles) infliximab infusion. Values are mean and SD of 11 independent experiments. **$p < 0.01$ by paired t test. D. Patients showed clinical improvement following the aforementioned infliximab infusion therapy. The disease activity of 11 RA patients before (closed bars) and 14 days after (hatched bars) infliximab infusion was estimated by DAS28-3. Values are mean and SD of 11 independent experiments. **$p < 0.01$ by paired t test.
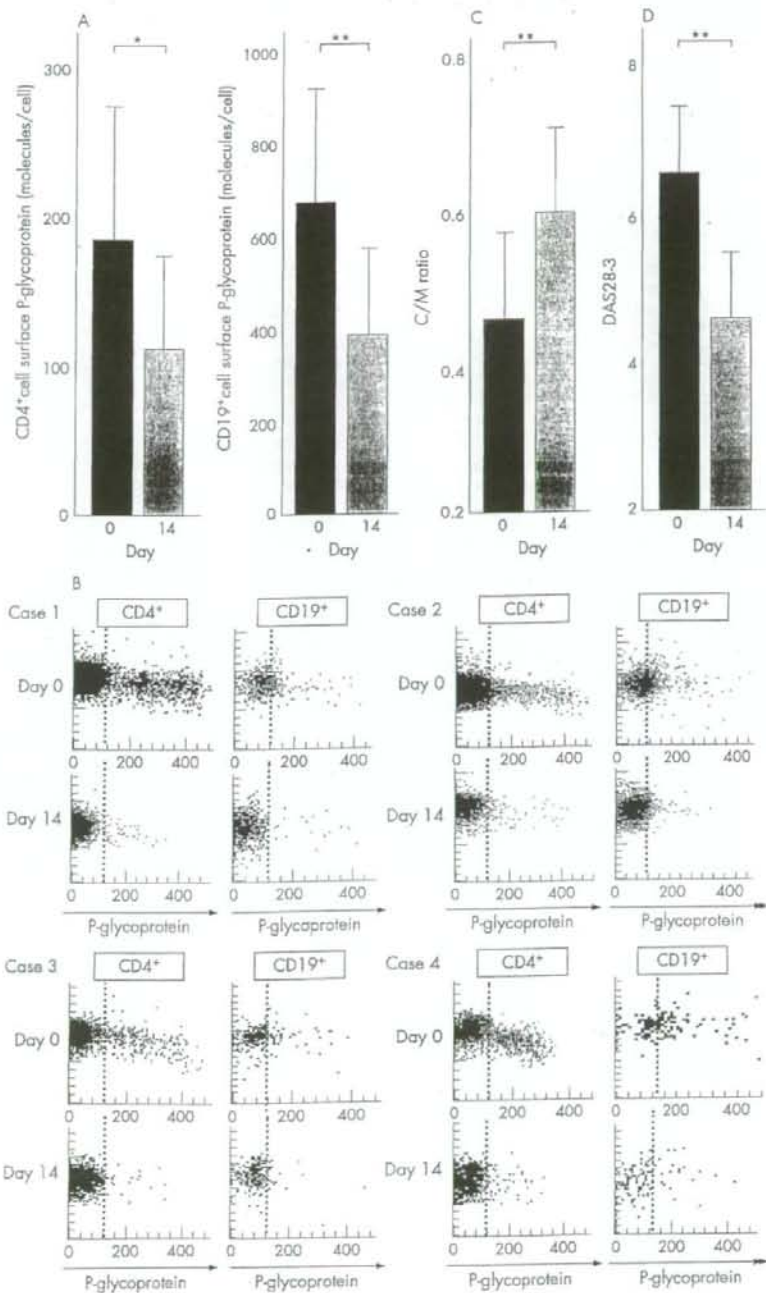
387

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Interaction of Galectin-9 With Lipid Rafts Induces Osteoblast Proliferation Through the c-Src/ERK Signaling Pathway

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ABSTRACT: Galectin-9 is a -galactoside–binding lectin expressed in various tissues, including bone. The role of galectin-9 in human osteoblasts, however, remains unclear. This study showed that galectin-9 interacts with lipid rafts and induces osteoblast proliferation through the c-Src/ERK signaling pathway.

Introduction: Galectin-9 is a -galactoside–binding lectin that modulates many biological functions by interacting with particular carbohydrates attached to proteins and lipids. However, the role of galectin-9 in bone metabolism and osteoblast proliferation remains unclear. This study investigated the effects of galectin-9 on osteoblast proliferation and its signaling mechanisms.

Materials and Methods: The effect of galectin-9 on osteoblast proliferation was tested by measuring the conversion of tetrazolium salt WST-8 to formazan. Protein phosphorylation was assayed by western blotting and confocal microscopy was used to localize lipid rafts.

Results: Galectin-9–induced proliferation of the obtained osteoblasts in a dose-dependent manner, whereas galectin-1, -3, and -4 did not. Galectin-9–induced phosphorylation of c-Src and subsequent ERK1/ERK2 in the osteoblasts. The galectin-9–induced phosphorylation and proliferation were inhibited by PP2, a selective inhibitor of c-Src. Galectin-9–induced clustering of lipid rafts detected by cholera toxin B (CTB; binding the raft-resident ganglioside GM1) using confocal microscopy. Cross-linking of the GM1 ganglioside with CTB by anti-CTB antibody–induced phosphorylation of c-Src, whereas disruption of galectin-9–induced lipid rafts by -methylcyclodextrin reduced c-Src phosphorylation and proliferation of the cells.

Conclusions: These results suggest that galectin-9, but not other galectins, induced proliferation of human osteoblasts through clustering lipid rafts on membrane and subsequent phosphorylation of the c-Src/ERK signaling pathway.

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Key words: galectin-9, osteoblast, proliferation, lipid rafts, c-Src

INTRODUCTION

V ARIOUS FACTORS STIMULATE osteoblast proliferation such as fibroblast growth factor-2 (FGF-2) and IGF-1,[1,2] although the precise regulatory mechanisms underlying this stimulation remain unknown. Lectins are carbohydrate-binding proteins that recognize carbohydrates attached to other proteins and lipids on cell surfaces and extracellular matrices. Animal lectins are grouped into several families[3] including galectins, which are defined by their ability to recognize -galactose and by a consensus amino-acid sequence.[4] Galectins contain conserved carbohydrate-recognition domains (CRDs) of 130 amino acids, which bind carbohydrate molecules.[5] Galectins modulate a variety of biological functions such as cell activation, pro-

liferation, adhesion, and apoptosis.[6–8] To date, 15 mammalian galectins have been identified and can be subdivided into three subgroups based on their structure[9]: one-CRD-type galectins, two-CRD-type galectins, and a chimeric-type galectin. The two-CRD-type galectins contain tandem repeats of two distinct CRDs.[10] Galectin-9 belongs to this group and is expressed in immune tissues such as thymus, lymph nodes, spleen, and bone marrow.[11] Galectin-9 confers various biological functions, including cell aggregation, chemoattraction of eosinophils, and apoptosis.[12–14] Relatively little is known about the role of galectins, particularly galectin-9, in bone metabolism, despite their expression at these sites.[15,16] This study examined the effect of galectin-9 on osteoblast proliferation and associated regulatory mechanisms. Because galectins bind cell surface glycoconjugates, we also studied the role of galectin-9 in the regulation of cell signaling through its effect on lipid rafts.

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278

MATERIALS AND METHODS

Purification of human osteoblastic cells

Human osteoblast-like cells were purified from metaphyseal trabecular bone of the proximal femur of five patients (four females and one male) who presented with osteoarthritis and underwent hip arthropathy according to the established procedures of Russell et al.[17] After removing pieces of cortical bone, articular cartilage, and soft connective tissue, the bone fragments were cut into small pieces and washed extensively. The bone explants were cultured in DMEM (GIBCO, Grand Island, NY, USA) containing 10% FCS (GIBCO) in 25-cm² culture flasks (Falcon, Lincoln Park, NJ, USA) under a 5% CO_2 atmosphere at 37°C. When cell monolayers reached confluence after 6–8 wk, the explants were removed, and the remaining cells were replated and cultured further to produce new cellular outgrowth and eventually a confluent monolayer of cells. The cells were trypsinized, passaged at 1 in 4, and recultured. The medium was changed twice a week, and the cells were used after three to seven passages.[18,19]

Characterization of human osteoblastic cells

Human osteoblast-like cells were grown to confluence in 60-mm dishes. The cells were washed three times with PBS and scraped into 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1% Triton X-100, 0.02% NaN₃, and 1 g/ml aprotinin. The lysates were homogenized by sonication for 20 s. Alkaline phosphatase (ALP) activity was measured in three different dishes derived from each culture using an ALP kit (Wako Pure Chemicals, Osaka, Japan). Osteocalcin released into the culture media was measured in triplicate using an appropriate ELISA kit (Bender Medsystems, Vienna, Austria). To normalize protein expression against total cellular protein, a fraction of the lysate solution was subjected to a Bradford protein assay. Cells in dishes were stimulated for 20 min with 100 nM human PTH(1-34) (Sigma, St Louis, MO, USA), and cAMP was measured after trichloracetic acid precipitation of the cell extracts using a cAMP assay kit (Cayman Chemicals, Ann Arbor, MI, USA). Results are expressed as picomoles of cAMP per milligram of cell protein.

Materials

Recombinant galectin-9 was prepared as described previously.[12] Recombinant human galectin-1, galectin-3, and galectin-4 were obtained from R&D Systems (Minneapolis, MN, USA). Horseradish peroxidase–conjugated goat anti-rabbit and horseradish peroxidase–conjugated horse anti-mouse secondary antibodies were obtained from Amersham Biosciences (Arlington Heights, IL, USA). Anti-c-Src antibody and anti-phospho-Src (Tyr⁴¹⁸) antibodies were purchased from Calbiochem (La Jolla, CA, USA). Anti-ERK and anti-phosphoERK antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). Alexa 488–labeled cholera toxin subunit B was obtained from Molecular Probes (Eugene, OR, USA), and methyl- -cyclodextrin (MCD) was purchased from Sigma. Various inhibitors of intracytoplasmic signaling including PP2 (Src-family kinase inhibitor), PP3 (Calbiochem; the inactive related compound of Src-family kinase inhibitor), PD98059 (MEK inhibitor), SB 202190 (p38 inhibitor), and SP 600125 (Cell Signaling Technologies; JNK inhibitor) were applied to each assay system, and all reagents were used at the indicated concentrations. At these concentrations, no inhibitor was cytotoxic to osteoblastic cells as confirmed by trypan blue staining.

Cell proliferation assay

Cell proliferation was assessed using a TetraColor One kit including WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium] and electron carrier mixture (Seikagaku, Tokyo, Japan). The WST-8 assay is based on the conversion of tetrazolium salt WST-8 to the highly water-soluble formazan.[20] Cells (1 × 10⁵) were seeded and incubated on a 96-well flat-bottomed plate (Iwaki) in DMEM containing 10% FCS in a final volume of 0.1 ml for 24 h at 37°C. They were treated with different agents for various time intervals. Ten milliliters of a solution containing 5 mM WST-8, 0.2 mM 1-methoxy-5-methylphenazium methosulfate, and 150 mM NaCl was added to each well. After incubation for 1 h at 37°C, the optical density of each well was measured on a microplate reader at 450 nm.

Confocal microscopy

Cells were plated in DMEM containing 10% FCS on coverslips in 60-mm culture plates and grown overnight at 37°C. After incubation with FGF-2, galectin-4, or galectin-9 for 3 min, the cells were washed with PBS and fixed in 3.7% formaldehyde for 15 min. The cells were washed five times with PBS before incubation with Alexa 488–labeled cholera toxin subunit B (CT-B) diluted 1:500 in PBS for 30 min. The cells were washed and examined on a Zeiss LSM5 PASCAL laser-scanning microscope (Carl Zeiss, Jena, Germany). In the GM1 ganglioside cross-linking experiment, cells were incubated for 15 min at 4°C with chilled medium containing Alexa 488–labeled CT-B. After washing three times, the cells were incubated for 15 min at 4°C with chilled medium containing anti-CT-B rabbit polyclonal antibody (Abcam, Cambridge, UK; 1:200).

Western blotting

Cells (1 × 10⁶) were cultured in DMEM containing 10% FCS in 6-well plates. The medium was changed when cell growth reached 80% confluence. After a further 12-h culture in DMEM containing 1% FCS, galectin-9 (100 nM) was added with or without various other agents. The cells were harvested and rinsed twice with PBS. The cell extracts were prepared with lysis buffer (20 mM Tris, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, and 10 g/ml leupeptin) and cleared by centrifugation at 12,000g for 15 min at 4°C. Total protein concentrations were determined (Bio-Rad, Hercules, CA, USA). The samples were subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Invitrogen, San Diego, CA, USA). Nonspecific binding sites were blocked by immersing the mem-
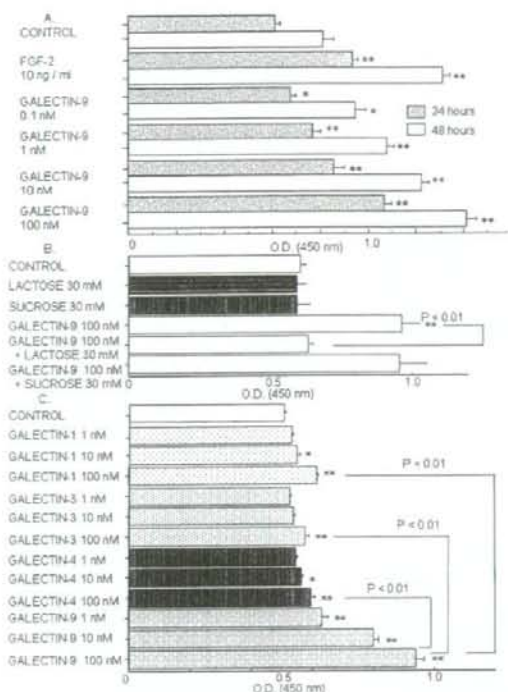
FIG. 1. Effects of galectin-9 on proliferation of human osteoblasts. (A) Galectin-9 increased proliferation of human osteoblasts in a time- and dose-dependent manner. Osteoblasts were incubated with various concentrations of galectin-9 or FGF-2 (10 ng/ml) for 24 (hatched bars) or 48 h (open bars). (B) Lactose inhibited galectin-9–induced proliferation of human osteoblasts. Cells were incubated with or without 30 mM lactose or 30 mM sucrose for 24 h, followed by stimulation with 100 nM galectin-9. (C) Galectin-9 induced stronger proliferation of human osteoblasts than galectin-1, galectin-3, or galectin-4. Cells were incubated with galectin-1, galectin-3, galectin-4, or galectin-9 at various concentrations and for the indicated time periods. Data are mean ± SD of five experiments in triplicate. *$p < 0.05$ and **$p < 0.01$ compared with control. The p values for analysis of difference between two values other than the control (open bars) are directly shown on the graphs.

branes in 5% skim milk in PBS for 1 h at room temperature, and the membranes were washed five times with PBS containing 0.1% Tween-20. The membranes were incubated overnight at 4°C with 1 g/ml primary antibody in PBS containing 0.1% Tween-20. After washing the membranes five times, the secondary antibody was added. Immunopositive bands were detected by chemiluminescence using the ECL reagent (Amersham Biosciences) and exposure to Hyperfilm-ECL (Amersham Biosciences).

Transfection of small interfering RNA

A small interfering RNA (siRNA) transfection kit specific for c-Src was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Osteoblasts were transfected

with c-Src siRNA or the control siRNA at 24 h before galectin-9 treatment according to the protocol recommended by the manufacturer.

Statistical analysis

All data were obtained from at least five independent experiments performed in triplicate. Results were expressed as mean ± SD. Statistical significance was computed by the unpaired Student's t-test. $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of human osteoblast-like cells in vitro

The cells obtained from human trabecular bone were plump and cuboidal with abundant cytoplasm and had a flattened polygonal shape with multiple spindle-shaped extensions (data not shown). The cells were subsequently characterized as osteoblast-like cells based on several features, including high intrinsic ALP activity, secretion of osteocalcin, and cAMP response to PTH. ALP levels were 81.2 ± 4.20 nmol/min/mg protein, and ELISAs showed the presence of osteocalcin in the culture supernatants (5.58 ± 1.32 ng/mg protein). Treatment with PTH increased the mean cAMP levels by 10.3-fold (3.11 ± 0.21–32.0 ± 1.21). These results indicated that osteoblasts were the primary cell type found in our bone-derived cell cultures from donors.

Galectin-9 induces proliferation of human osteoblasts

Initially, galectin-9 significantly induced proliferation of the obtained human osteoblasts in a concentration-dependent manner at 24 and 48 h after stimulation (Fig. 1A). The proliferative activity induced by galectin-9 was comparable to that of 10 ng/ml of FGF-2, a well-known
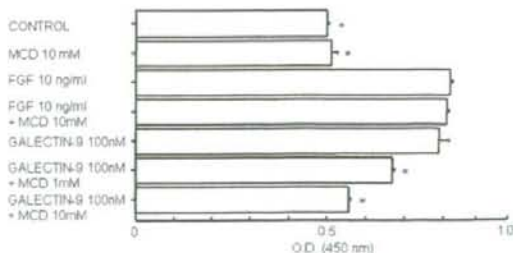
FIG. 2. Disruption of lipid rafts inhibited galectin-9–induced proliferation of human osteoblasts. Cells were pretreated with various concentrations of MCD for 30 min before exposure to galectin-9 or FGF-2 (10 ng/ml). Cells were washed to remove MCD and incubated with 100 nM galectin-9 for 24 h. Data are mean ± SD of five experiments in triplicate. *$p < 0.01$ compared with galectin-9 without MCD.
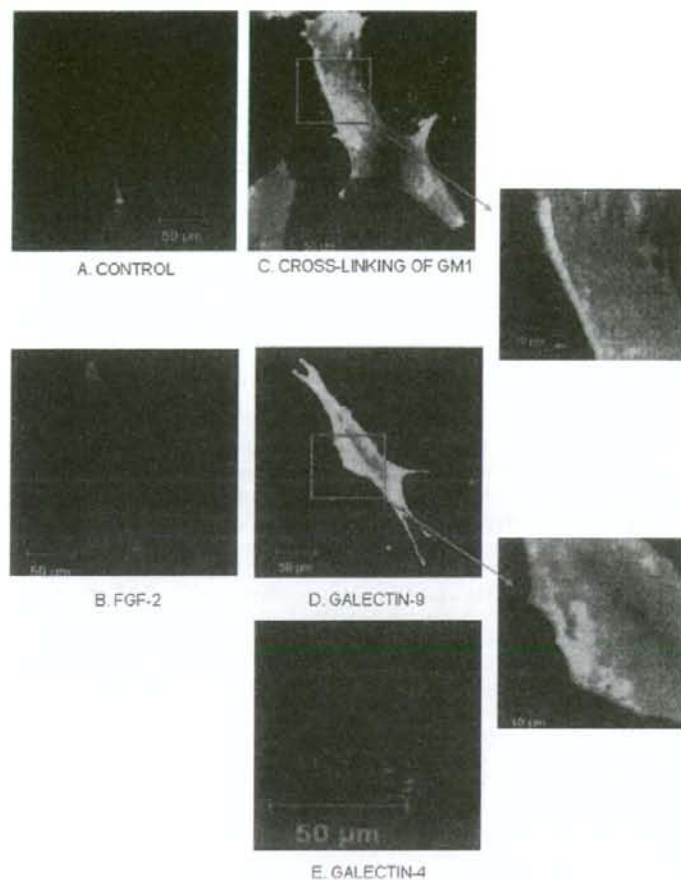
FIG. 3. Galectin-9 induces clustering of lipid rafts on human osteoblasts. Cells were incubated with 100 nM galectin-9, 100 nM galectin-4, or FGF-2 (10 ng/ml) for 3 min and stained with Alexa 488–labeled cholera toxin subunit B. In the GM1 cross-linking experiment, cell were incubated for 15 min with anti-CT antibody after incubation with Alexa 488–labeled cholera toxin subunit B for 15 min. Magnification, ×400 for all panels. Scale bars, 50 m. (A) Control. (B) FGF-2. (C) Cross-linking of GM1. (D) Galectin-9. (E) Galectin-4. C and D were represented at higher magnification (×800). This figure is representative of three independent experiments.

potent stimulator for the osteoblasts and was observed in the lower concentration of galectin-9, 0.1 nM. We also monitored the proliferative index, as determined by proliferation cell nuclear antigen (PCNA)-positive cells, and results were as follows: control, $16.7 \pm 1.9\%$; galectin-9 1 nM, $24.1 \pm 4.2\%$; galectin-9 10 nM, $28.7 \pm 1.9\%$; galectin-9 100 nM, $38.3 \pm 0.8\%$. Furthermore, lactose (30 mM), but not sucrose, inhibited galectin-9–induced proliferation of the osteoblasts, implying that the activity makes sense characteristics of molecules of the galectin family (Fig. 1B). Although galectin-9–induced proliferation of osteoblasts showed marked concentration dependency, galectin-1, galectin-3, and galectin-4, even at a high dose (100 nM), only slightly or marginally stimulated osteoblast proliferation in this study (Fig. 1C), suggesting that galectin-9 is a more potent regulator of osteoblast proliferation than others.

### Disruption of lipid rafts inhibits galectin-9–induced proliferation of human osteoblasts

MCD extracts and sequesters cellular cholesterol and thus disrupts lipid rafts in cell membranes,[21,22] providing a useful pharmacological tool for evaluating the cholesterol-rich membrane domains such as lipid rafts. The aim of using MCD in our studies was to inhibit lipid raft function. To study whether galectin-9 induces osteoblast proliferation through formatting lipid rafts, we pretreated osteoblasts for 30 min with MCD before exposing the cells to galectin-9 or FGF-2. The 96-well plate was washed with medium to remove the MCD and incubated with galectin-9 or FGF-2 for a further 24 h. Although incubation of osteoblasts with various concentrations of MCD did not alter cell morphology or integrity (data not shown), MCD inhibited the galectin-9–induced osteoblast proliferation in a dose-dependent manner. In addition, neither spontaneous nor FGF-2–induced proliferation was influenced by lipid raft integrity (Fig. 2).

Galectin-9–induced clustering of membrane lipid rafts

Confocal microscopic analysis indicated that the clustering of lipid rafts was clearly induced in osteoblasts treated with galectin-9 as assessed by the localization of Alexa 488–

A

B

C
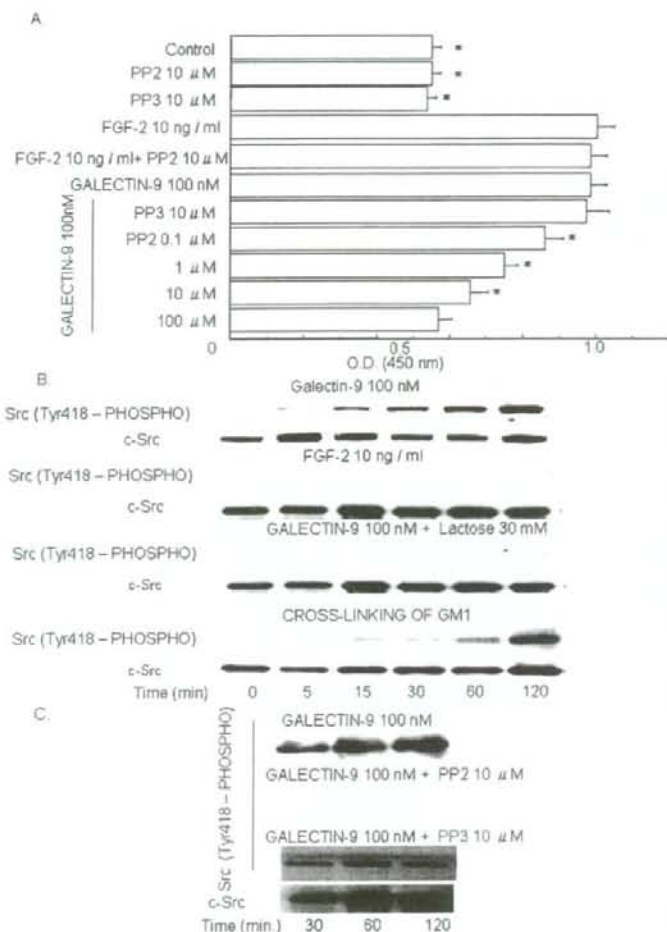
FIG. 4. Effects of c-Src kinase on proliferation of human osteoblasts. (A) PP2 inhibited proliferation of human osteoblasts. Cells were incubated with various concentrations of PP2 or PP3, followed by 100 nM galectin-9 or FGF-2 (10 ng/ml) for 24 h. Data are mean $\pm$ SD of five experiments in triplicate. $*p <$ 0.01 compared with galectin-9 without PP2. (B) Galectin-9 induced activation of c-Src kinase in human osteoblasts. Cell lysates were prepared from human osteoblasts, stimulated by FGF-2 (10 ng/ml), 100 nM galectin-9 with or without 30 mM lactose, or cholera toxin subunit (CT-B) and anti-CT-B antibody for the indicated time intervals (0, 5, 15, 30, 60, and 120 min), followed by Western blotting with anti-c-Src antibody or anti-phospho-Src (Tyr[418]) antibody. This figure is representative of three independent experiments. (C) PP2 inhibited c-Src phosphorylation in human osteoblasts. Cell lysates were prepared from cells stimulated with 100 nM galectin-9 and 10 M of PP2 or PP3, followed by Western blotting with anti-c-Src antibody or anti-phospho-Src (Tyr[418]) antibody. This figure is representative of three independent experiments.

labeled cholera toxin subunit B (CTB), which is known to bind the raft-resident ganglioside GM1 and is used as a representative raft marker[23,24] (Fig. 3). However, these clusters of CTB and GM1 were neither observed in the vehicle control nor in cells treated with FGF-2. Also galectin-4, which has a similar structure to galectin-9, failed to create clustering of CTB/GM1, implying that the galectin-9 uniquely induced lipid-raft clustering and it might initiate downstream signaling to trigger osteoblast proliferation.

Src-family kinase is involved in galectin-9–mediated signaling in human osteoblasts

Because galectin-9 is known to induce phosphorylation of Src-family proteins, which is concentrated within lipid rafts,[25] we next assessed which signaling pathway is involved in galectin-mediated signaling in human osteoblasts, shedding light on Src-family tyrosine kinase. As shown in Fig. 4A, galectin-9 induced proliferation of the human osteoblasts, but PP2, a selective and potent inhibitor of c-

Src,[26,27] but not by PP3, its inactive related compound inhibited galectin-9–induced proliferation of the cells in a concentration-dependent manner. Furthermore, after galectin-9 stimulation, the immunoblot-staining in total cell lysates using a specific anti-phospho-Src (Tyr[418]) antibody was rapidly induced within 5 min, and the staining was gradually increased in a time-dependent manner by 120 min after stimulation (Fig. 4B). The galectin-9–induced phosphorylation of c-Src (Tyr[418]) was inhibited by the addition of lactose to the culture, and FGF-2 did not induce the phosphorylation. Also, the addition of PP2, but not PP3, inhibited the galectin-9–induced phosphorylation of c-Src (Tyr[418]) (Fig. 4C). Moreover, cross-linking of the raft-resident GM1 ganglioside with CTB by anti-CTB antibody induced phosphorylation of c-Src (Tyr[418]) in osteoblasts by the similar time-course as galectin-9 did (Fig. 4B). Furthermore, the knockdown of c-Src by the transfection of a siRNA complementary to c-Src into human osteoblasts, in which endogenous c-Src protein expression reduced (Fig.
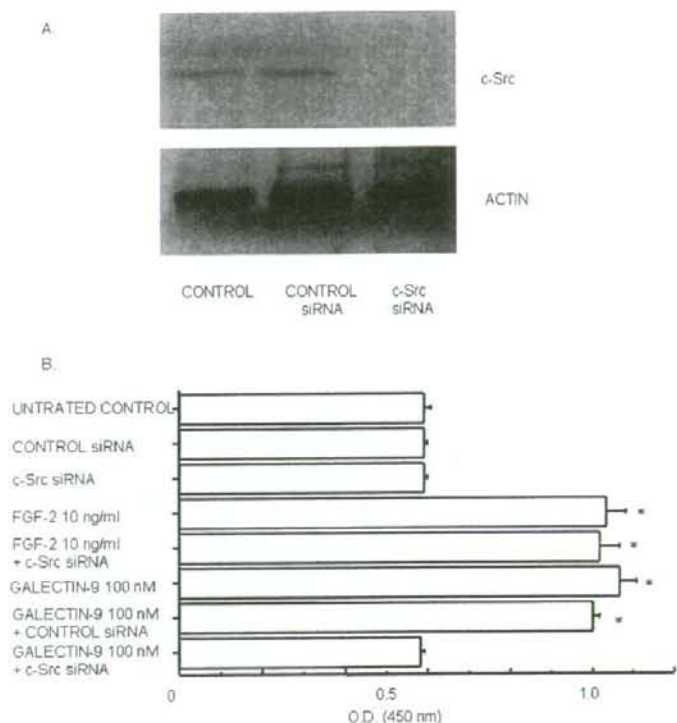
FIG. 5. Effects of c-Src siRNA on proliferation of human osteoblasts. (A) Cell lysates were prepared from cells treated with the control or c-Src siRNA for 24 h, followed by Western blotting with anti-c-Src antibody. (B) Cells were incubated with the control or c-Src siRNA for 24 h, followed by 100 nM galectin-9 or FGF-2 (10 ng/ml). Data are mean ± SD of five experiments in triplicate. *$p < 0.01$ compared with galectin-9–untreated siRNA.

5A), inhibited the proliferation of the cells induced by galectin-9 but not by FGF-2 (Fig. 5B). Taken together, these results suggest that galectin-9 specifically phosphorylated a certain tyrosine residue of a c-Src family protein through clustering a lipid raft on membrane, which led to the proliferation of the osteoblasts.

Galectin-9 phosphorylates ERK in human osteoblasts

We next assessed the downstream signaling pathway of c-Src kinase in the osteoblasts. The addition of PD 98059, a MEK inhibitor, to the culture medium during a 24-h incubation period did not affect cell morphology as examined by light microscopy, but blocked the galectin-9–induced osteoblast proliferation (Fig. 6A). However, neither dimethyl sulfoxide (DMSO) vehicle alone, nor SB 202190 or SP 600125, mitogen-activated protein kinase (MAPK) inhibitors, altered the mitogenic activity of galectin-9. Furthermore, after galectin-9 stimulation, phophorylation of both ERK1 and ERK2 was rapidly induced within 5 min, and its phosphorylation become clearer at 30–120 min after stimulation, although total levels of ERK proteins did not change during the 180-min incubation (Fig. 6B). Also, the addition of PP2, but not PP3, inhibited the galectin-9–induced phosphorylation of ERK1/ERK2 at 60 min after galectin-9 stimulation in a dose-dependent manner (Fig. 6C). These findings indicate that the ERK signaling effect was downstream of c-Src kinase.

Raft-disrupting agent inhibits c-Src kinase activation

Finally, to study the possible association of c-Src with lipid rafts, we measured galectin-9–induced phosphorylation of c-Src (Tyr^{418}) after pretreatment with various concentrations of MCD and showed an inhibitory effect (Fig. 7). This result indicates that c-Src kinase is associated with lipid rafts.

DISCUSSION

In this study, we showed that galectin-9 induced osteoblast proliferation through interaction with lipid rafts. To our knowledge, such an effect for galectin-9 through interaction with lipid rafts has not been reported previously in osteoblasts. Interestingly, galectin-9 induced osteoblast proliferation through pathways different from those stimulated by FGF-2. Previous studies implicated galectin-9 in apoptosis of certain tumor cell lines such as Jurkat, MOLT-4, BALL-1, THP-1, and HL-60[12,13] and indicated that galectin-9 may have different effects on different cells. In this study, disruption of lipid rafts by MCD inhibited osteoblast proliferation and galectin-9–induced clustering of lipid rafts. Lipid rafts are liquid-ordered membrane microdomains with a unique protein and lipid composition found on the plasma membrane of most mammalian cells. A large number of signaling molecules are concentrated within these domains, which might function as signaling centers to
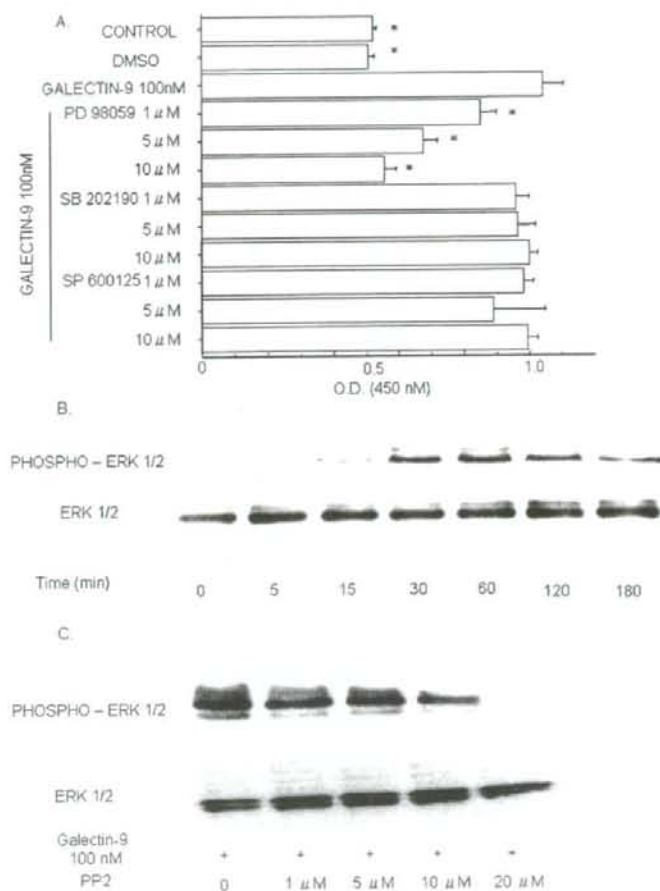
FIG. 6. Effects of ERK1/2 on proliferation of human osteoblasts. (A) PD98059 inhibited proliferation of human osteoblasts. Cells were incubated with various concentrations of PD98059, SB202190, or SP600125, followed by 100 nM galectin-9 for 24 h. Data are mean ± SD of five experiments in triplicate. *$p < 0.01$ compared with galectin-9 without PD98059. (B) Galectin-9 induced ERK 1/2 phosphorylation in human osteoblasts. Cell lysates were prepared from human osteoblasts treated with 100 nM galectin-9 for the indicated time intervals (0, 5, 15, 30, 60, 120, and 180 min), followed by Western blotting with an anti-phospho-ERK1/2 antibody. This figure is representative of three independent experiments. (C) c-Src activation by galectin-9 induced ERK1/2 phosphorylation. Cell lysates were prepared from cells treated with 100 nM galectin-9 and various concentrations of PP2 for 180 min, followed by Western blotting with anti-phospho-ERK1/2 antibody. This figure is representative of three independent experiments.

facilitate efficient and specific signal transduction.[28] Cross-linking or clustering of specific biological components such as receptors is an indispensable element of many membrane-associated processes. The correlation between protein cross-linking and membrane domain formation is particularly well established for immune-recognition receptor signaling in B, T, and mast cells.[29–32] In this study, galectin-9 specifically induced clustering of lipid rafts, and the activity of galectin-9 seemed more potent than that of other galectin family members. Biochemical analysis of purified lipid rafts from different cell types showed a striking concentration of signaling molecules.[33–36] Clustering of lipid rafts in response to certain stimuli could therefore rapidly create higher-order signaling complexes that may amplify signals or enhance cross-talk between related signaling pathways.[28] Because galectin-9 induced clustering of lipid rafts, clustering of lipid rafts is thought to be an important trigger of osteoblast proliferation induced by galectin-9. Furthermore, clustering of lipid rafts on osteoblasts may subsequently initiate the activation of downstream signaling pathways.

Our results also showed that interaction of galectin-9 with lipid rafts induced osteoblast proliferation through c-Src/ERK signaling. c-Src is localized on the plasma membrane and is often associated with lipid rafts,[37–39] as well as osteoblast proliferation.[40] Intramolecular interactions between the SH2 domain of Src family kinases and the phosphorylated tail maintain their inactive conformation. The displacement of this interaction by SH2-binding phosphotyrosines in receptors activates Src kinases.[41,42] Subsequently, Tyr-418 in the Src tyrosine kinase domain is, autophosphorylated.[42] Lactose, a saccharide ligand recognized by galectins, inhibited the galectin-9–induced c-Src activation after only a few minutes in our study. Taken together, these findings suggest c-Src activation to be a direct effect of galectin-9 rather than one of endogenous factors induced by galectin-9. The ERK signaling pathway is downstream of Src kinase activation,[43,44] and indeed here, Src inhibition reduced ERK phosphorylation.

Galectin-9–induced proliferation was stronger than that induced by other galectins. This might be because of the enhanced ability of galectin-9 to induce clustering of lipid rafts. Galectins are present both inside and outside cells and function at both sites. Extracellularly, galectins bind cell
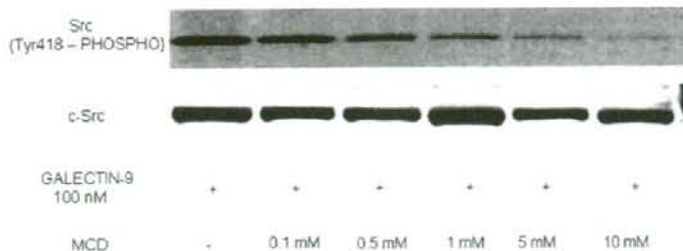
FIG. 7. Raft-disrupting agent inhibits c-Src kinase activation. Cell lysates were prepared from cells treated with 100 nM galectin-9 and various concentrations of MCD for 120 min, followed by Western blotting with anti-c-Src or anti-phospho-Src (Tyr[418]) antibody. This figure is representative of three independent experiments.

surface glycoconjugates containing suitable galactose-containing oligosaccharides. Because galectins can bind either bivalently or multivalently, they can cross-link cell surface glycoconjugates, and like many other receptor–ligand systems, can trigger a cascade of transmembrane signal events.[10] Whereas the galectin-9 ligand was not identified in this study, lipid rafts are associated with various glycoproteins and glycolipids. Other investigators have reported the binding of galectin-1 to GM1, a component of lipid rafts.[45] Galectin-9 might therefore induce lipid-raft clustering by binding glycoconjugates such as GM1 on the lipid rafts.[46] Galectin-9 is a tandem-repeat type galectin,[5,6] containing two distinct CRDs in tandem and connected by a linker of up to 70 amino acids. These galectin-9–specific structural features allow easy recognition, binding, and cohesion to glycoconjugates on lipid rafts. Galectin-9 can also bind multivalently, and this may facilitate the lipid raft clustering. In addition, recent reports cited galectin-4 as a strong organizer/stabilizer of lipid rafts.[47,48] Similar properties could now be attributed to galectin-9. However, galectin-4 did not induce osteoblast proliferation and lipid rafts clustering. In different cell types galectin-4 may have different effects. These effects in osteoblasts are thought to be particular effects of galectin-9 and not induced by other different galectins.

In conclusion, we showed that galectin-9 induces osteoblast proliferation through the c-Src/ERK signaling pathway through interaction with lipid rafts. We propose that clustering of lipid rafts induced by galectin-9 may be an important trigger for osteoblast proliferation through c-Src/ERK signaling. Further understanding of galectin-9 function should broaden our knowledge of its role in bone metabolism.

ACKNOWLEDGMENTS

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Bolus infusion of human urinary trypsin inhibitor improves intractable interstitial pneumonia in patients with connective tissue diseases

S. Tsujimura, K. Saito, S. Nakayamada and Y. Tanaka

Objective. Interstitial pneumonia (IP) associated with CTDs often progresses despite conventional immunosuppressive treatment. We investigated the efficacy of human urinary trypsin (UT) inhibitor (ulinastatin) on refractory IP.

Methods. Five patients with IP received UT inhibitor (3×10^5 U) infusion into the internal jugular vein, three times in a single day. The response to this therapy was assessed clinically and by chest CT, PaO_2 and serum KL-6. The kinetics of UT inhibitor was determined in arterial blood. We measured serum levels of monocyte chemotactic protein-1 and TGF-β1, which are thought to be involved in the pathogenesis of IP.

Results. Serum concentrations of UT inhibitor increased immediately to >150 U/ml after infusion of 3×10^5 U of UT inhibitor. The treatment resulted in clinical and radiological improvements in four patients, and allowed reduction of oxygen therapy following improvement of hypoxaemia within 1 month. UT inhibitor decreased serum levels of KL-6 in all patients and had no adverse effects. MCP-1 and TGF-β1 concentrations were higher in the patients than in normal subjects, and infusion of 3×10^5 U of UT reduced the concentrations within 3 h of infusion.

Conclusion. UT inhibitor bolus infusion therapy is a potentially useful therapeutic strategy for intractable IP based on the different mechanism of action relative to conventional immunosuppressive therapy and lack of serious treatment-related adverse effects.

KEY WORDS: Human urinary trypsin inhibitor, Interstitial pneumonia, Connective tissue diseases.

Introduction

Interstitial pneumonia (IP) is a serious complication in patients with systemic autoimmune diseases, and sometimes progresses rapidly causing death in some patients [1, 2]. Although immunosuppressive-cytotoxic agents, such as AZA, CSA, tacrolimus and cyclophosphamide, are used in combination with corticosteroids in patients with refractory IP [3–5], their efficacy for IP is controversial [2, 6]. In addition, these agents are not always tolerated because of various adverse effects including opportunistic infections [7–9]. In such cases, intensive immunosuppressive therapy cannot be continued, and measures to treat patients with repeated opportunistic infection are needed.

The pathological process of IP is accelerated by various inflammatory factors at the loci of interstitial inflammation. Ulinastatin, a human urinary trypsin (UT) inhibitor, is a natural inhibitor of protease and frequently used for the treatment of shock [10] and acute pancreatitis [11]. Since UT inhibitor is also known to inhibit various inflammatory factors associated with the development and progression of IP, such as cytokines [12], oxygen radicals [13] and adhesion molecules [14], it is possible that UT inhibitor could be therapeutically useful against active IP. In fact, we have reported that bolus infusions of UT inhibitor improved severe IP in MCTD [15]. However, the efficacy of UT inhibitor in IP with CTDs has not been established. In present study, we used UT inhibitor in bolus infusion to treat five patients with active IP who did not respond to immunosuppressive therapy or developed adverse effects, and evaluated the efficacy and safety of such treatment.

Patients and methods

Patients

Five patients with IP complicating systemic autoimmune diseases [one with MCTD, two with SSc, one with microscopic polyangiitis (MPA), and one with DM], who were admitted to our university hospital between July 2002 and June 2004, were included in this study (Table 1). The diagnosis of MCTD was based on the criteria described by Alarcon-Segovia and Villarreal [16]. SSc was diagnosed according to the ACR criteria [17]. The Chapel Hill nomenclature was used to define MPA [18]. DM was diagnosed according to the criteria of Bohan and Peter [19]. IP in the five patients fulfilled all the following criteria: (i) IP was associated with CTDs, not with drugs or infection; (ii) IP was progressively assessed by symptoms including dyspnoea, progressive decrease in partial pressure of oxygen in arterial blood (PaO_2) and worsening of chest radiographic findings; and (iii) complications apart from IP were controllable but IP was not suppressed sufficiently by immunosuppressants and/or could not be treated further with immunosuppressants due to adverse effects. IP in four patients progressed in spite of intensive immunosuppressive treatment and resulted in severe *Candida* pneumonia (Patient 1), acute pancreatitis (Patient 2), *Pneumocystis* pneumonia (PCP) (Patient 3) and pandemic cingulum (Patient 5). IP in Patient 4 could not be resolved with CSA plus intravenous cyclophosphamide pulse therapy (IVCY), and finally resulted in acute exacerbation of IP. The use of UT inhibitor bolus infusion therapy was approved by the ethics committee of our institution and informed consent was obtained from the patients.

Assessment of IP

The severity of IP was assessed by chest CT, PaO_2 and serum levels of KL-6. KL-6 is a mucinous glycoprotein expressed on type II pneumocytes and used clinically as a marker of IP in various collagen diseases and of pulmonary interstitial damage [20, 21]. Serum KL-6 levels increase with deterioration of IP, while successful treatment of IP results in significant falls in these levels [21]. Serum KL-6 concentrations were measured by electrochemiluminescence immunoassay (Eisai, Tokyo, Japan).

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907

TABLE 1. Characteristics of five patients with interstitial pneumonia

Characteristics	Patient number				
	1	2	3	4	5
Age (yrs)	40	69	78	47	50
Sex	F	F	F	F	F
Diagnosis	MCTD	SSc	MPA	SSc	DM
Disease duration (yrs)	5	9	8	2	6
LDH (IU/ml)	160	222	227	279	295
KL-6 (U/ml)	8253	702	819	3690	1775
PaO_2 (torr)[a]	55	74	56	57	74
DL_{co} (ml/min/torr)	7.59	ND	ND	6.61	4.99
%VC	71	48	ND	75	57
Treatments prior to UT inhibitor					
Oral PSL;					
Duration (yrs)	6	8	7	–	6
Maximum dose (mg/kg/day)	1.0	0.5	1.0	–	1.0
Immunosuppressants	mPSL pulse (3 times), CSA (3.5 yrs), IVCY (11 times)	CSA (2 weeks)	mPSL pulse (twice), CPA (3 yrs)	CSA (1 yr), IVCY (3 times), D-pc (1 yr)	CSA (2.5 yrs), IVCY (once)
Adverse effects					
Multiple compression fractures	+	–	+	–	+
Opportunistic infections	*Candida*	–	*Pneumocystis*	–	CMV, VZV
Others	Diabetes mellitus, Renal dysfunction due to CSA	Pancreatitis due to CSA	–	–	Bilateral femoral head necrosis, Erythroderma due to IVCY

[a]Data are PaO_2 of Cases 1, 3, 4 and 5 on breathing room air and Case 2 breathing 1.0 l/min of oxygen. LDH: lactate dehydrogenase (normal range: 120–230 IU/l); KL-6 (normal range: 105–401 U/ml); DL_{co}: diffusing capacity for carbon monoxide; ND: not done; %VC: the percent vital capacity; mPSL pulse: methylprednisolone pulse therapy; CPA: oral cyclophosphamide; D-pc: D-penicillamine; *Candida*: fungal pneumonia with *Candida albicans*; VZV: varicella zoster virus.

UT inhibitor bolus infusion therapy

UT inhibitor (ulinastatin, Miracrid®, Motida Ltd, Japan) was infused into the internal jugular vein, rather than a peripheral vein, using a central venous catheter in order to obtain a high UT inhibitor concentration in the lung. A single course of UT inhibitor bolus infusion therapy consisted of bolus infusion of 3×10^5 U of UT inhibitor repeated three times at 5-h intervals (total dose: 9×10^5 U/day). After the bolus infusion, the dose of prednisolone (PSL) was not changed but no new immunosuppressants were added for 1 month.

Kinetics of UT inhibitor concentration in arterial blood

We measured the serum concentrations of UT inhibitor by radioimmunoassay (SBS, Kanagawa, Japan) in arterial blood samples obtained from the brachial artery of three patients to evaluate UT inhibitor concentration in the pulmonary circulation over a period of 60 min after initial bolus infusion of 3×10^5 U of UT inhibitor.

Measurements of monocyte chemotactic protein-1 and TGF-β1

We collected arterial blood samples from the brachial artery of six normal volunteers and patients to measure the levels of monocyte chemotactic protein (MCP-1) and TGF-β1 in the pulmonary circulation. Informed consent was obtained from all the donors who were enrolled in the study. The serum was separated rapidly from the collected arterial blood (within 15 min after collection), and MCP-1 and TGF-β1 were measured with an enzyme immunoassay system (SBS).

Statistical analysis

Values are expressed as mean ± s.d. Student's *t*-test was used to compare data between the two groups. A *P*-value <0.05 denoted the presence of statistically significant difference.

Results

Kinetics of UT inhibitor concentration in arterial blood

Arterial blood samples were collected from the brachial artery over a period of 60 min and the serum concentrations of UT inhibitor were measured. UT inhibitor concentrations were >150 U/ml at 20 min and remained at more than 100 U/ml at the end of 60 min after a single bolus infusion of 3×10^5 U of UT inhibitor (Fig. 1).

Clinical efficacy of UT inhibitor bolus infusion therapy

Patient 1 was diagnosed as MCTD with IP, and intensive immunosuppressive treatment with high-dose corticosteroids and CSA or repeated IVCY did not resolve the progressive IP for 7 yrs, and finally resulted in severe *Candida* pneumonia. Infusion of amphotericin B improved *Candida* pneumonia, but hypoxaemia worsened with progression of CT-documented MCTD-related IP. However, additional immunosuppressive therapy was considered inappropriate in the presence of the fungal infection. Accordingly, we used UT inhibitor bolus infusion therapy. This resulted in the attenuation of the Velcro rales and improvement of ground-glass attenuation on the chest X-ray. Two weeks after a single course of UT inhibitor bolus infusion therapy, the patient showed relief from dyspnoea and a decrease in all-day persistent cough to a few episodes per day. Moreover, serum level of KL-6 fell from 8253 to 5841 U/ml and PaO_2 increased to 80 mmHg. Six months after nine courses of UT inhibitor bolus infusion therapy once every month, she was maintained on only 7 mg/day of PSL when KL-6 decreased to 938 U/ml (Fig. 2A) [15].

Patient 2 was admitted to our hospital for exacerbation of IP after 8-yr corticosteroid therapy for idiopathic IP, combined with 2-yr home oxygen therapy. After admission, she was diagnosed as SSc with IP, based on the presence of RP, severe skin sclerosis and the gastrointestinal tract manifestations. Despite the addition of CSA, the ground-glass attenuation increased on chest X-ray and CT, and then she developed acute pancreatitis. Pancreatitis responded well to infusion of antibiotics and withdrawal of CSA. UT inhibitor bolus infusion therapy was applied for progression of IP. The treatment resulted in attenuation of the
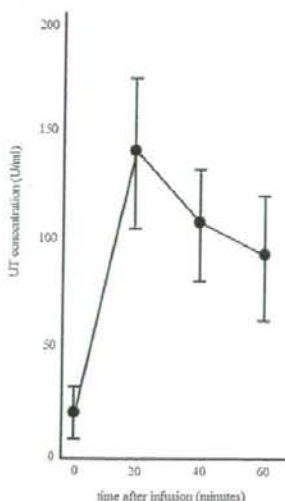
ground-glass appearance, improvement of PaO_2, allowing a reduction in oxygen inhalation to 1.0 l/min. After two courses of UT inhibitor bolus infusion therapy, KL-6 decreased to 403 U/ml. She was maintained on only 5 mg/day of PSL at discharge from the hospital (Fig. 2B).

Patient 3 was diagnosed as MPA, based on IP, rapid progressive glomerulonephritis and high titre of myeloperoxidase-specific ANCA. Despite treatment with oral cyclophosphamide, high-dose corticosteroids and methylprednisolone pulse infusion, IP recurred several times during a period of 8 yrs and finally resulted in PCP. Although PCP responded to pentamidine, MPA-related ground-glass attenuation on CT was exacerbated. A single course of UT inhibitor bolus infusion therapy resulted in recovery of PaO_2 and relief from dyspnoea, allowing withdrawal of oxygen therapy. Subsequent follow-up showed diminished uptake on gallium citrate uptake scintigraphy, thus allowing withdrawal of oral cyclophosphamide. Maintenance treatment on discharge was 2 mg/day PSL (Fig. 2C).

Patient 4 was diagnosed as SSc with IP. IP and skin sclerosis progressed gradually in spite of treatment with CsA and IVCY for 15 months. The patient was admitted to our hospital for treatment of acute exacerbation of IP. UT inhibitor bolus infusion therapy was immediately initiated. Three days after infusion, dyspnoea disappeared on rest, PaO_2 began to improve allowing reduction of oxygen inhalation (PaO_2 90 mmHg on 0.5 l/min of oxygen inhalation), serum lactate dehydrogenase diminished to the normal range and KL-6 decreased (Fig. 2D). The patient was moved to another hospital for treatment of progressive skin sclerosis with haematopoietic stem cell transplantation.

Patient 5 was diagnosed as DM with IP. IP recurred many times despite treatment with high-dose corticosteroids combined with CsA and pulse methylprednisolone for 5 yrs. The patient developed CMV infection, severe hypertension and bilateral femoral head necrosis. CMV infection necessitated withdrawal of CsA and ganciclovir infusion, but IP showed central exacerbation, followed by the development of pandemic cingulum. UT inhibitor bolus infusion therapy combined with aciclovir was added and PSL dose tapered. This regimen resulted in attenuation of Velcro rales, gradual resolution of the ground-glass

abnormality on the chest X-ray, improvement of PaO_2, and gradual healing of pandemic cingulum. Although IP tended to recur upon tapering PSL to 7.5 mg/day, repeated UT inhibitor bolus infusion therapy suppressed exacerbation of IP and allowed the use of a maintenance PSL dose of only 5 mg/day (Fig. 2E).

Although patient 4 was moved to another hospital after only a single course of UT inhibitor bolus infusion therapy, the other four patients received UT inhibitor bolus infusion therapy repeatedly once every month, and the repeated infusion resulted in further improvement of IP despite the tapering of corticosteroids and/or withdrawal of other immunosuppressants (Fig. 2A–C, E). Furthermore, long-term repetitive UT inhibitor bolus infusion therapy resulted in marked improvement of IP in Patient 1 over 8 months. Interestingly, exacerbation of IP was noted after withdrawal of UT inhibitor bolus infusion therapy, but re-infusion resulted in marked improvement of IP again (Fig. 2A) [15]. There were no adverse effects related to UT inhibitor bolus infusion therapy in all five patients.

Effects of UT inhibitor bolus infusion therapy on laboratory and imaging findings

Table 2 summarizes the effects of UT inhibitor bolus infusion therapy on laboratory and imaging findings at 1 month after the course of treatment. Dyspnoea and Velcro rales improved in four out of five patients, PaO_2 increased in all patients (Fig. 3) and flow rate of oxygen could be reduced in all patients. Furthermore, serum levels of KL-6 decreased in all patients after a single course of UT inhibitor bolus infusion therapy. The PaO_2 and serum levels of KL-6 improved significantly relative to the corresponding values before infusion (Fig. 3).

Figure 4 shows CT findings of the five patients before and after treatment. The findings improved markedly in Patients 1, 3 and 5 after UT inhibitor bolus infusion therapy. Furthermore, the density of ground grass attenuation decreased gradually in Patient 4, while pulmonary fibrosis in Patient 2 was almost unchanged at 1 month after a single course of UT inhibitor bolus infusion therapy.

Effects of UT inhibitor on MCP-1 and TGF-β1

Since UT inhibitor is known to have anti-inflammatory and organ-protective effects through the inhibition of various inflammation-related mediators, we examined whether UT inhibitor bolus infusion influenced the production of MCP-1 and TGF-β1, which are both reported to be associated with the progression of IP [22, 23]. Before treatment, serum concentrations of MCP-1 and TGF-β1 were significantly higher in patients with IP than the normal control (Fig. 5). However, the initial bolus infusion of 3×10^5 U UT inhibitor resulted in a significant decrease in serum MCP-1 level measured at 3 h after infusion and also tended to decrease serum TGF-β1 concentration albeit insignificantly.

Discussion

Progressive IP is a life-threatening manifestation of autoimmune disease and often intractable despite concomitant therapy with high-dose corticosteroids and immunosuppressants [1, 2]. In addition, repeated intensive immunosuppressive therapy including high-dose corticosteroids often causes treatment-related severe adverse effects such as opportunistic infections like fungal, pneumocystis and CMV pneumonia [7–9]. Once patients develop such complication of severe infections, the use of additional immunosuppressants is inappropriate even when IP is not controlled well. We presented three patients who showed resistance to various combination therapies of corticosteroid and immunosuppressants including repeated IVCY, and finally developed severe opportunistic pneumonias. However, UT inhibitor bolus infusion therapy successfully improved refractory IP, without serious adverse effects such as bone marrow suppression,
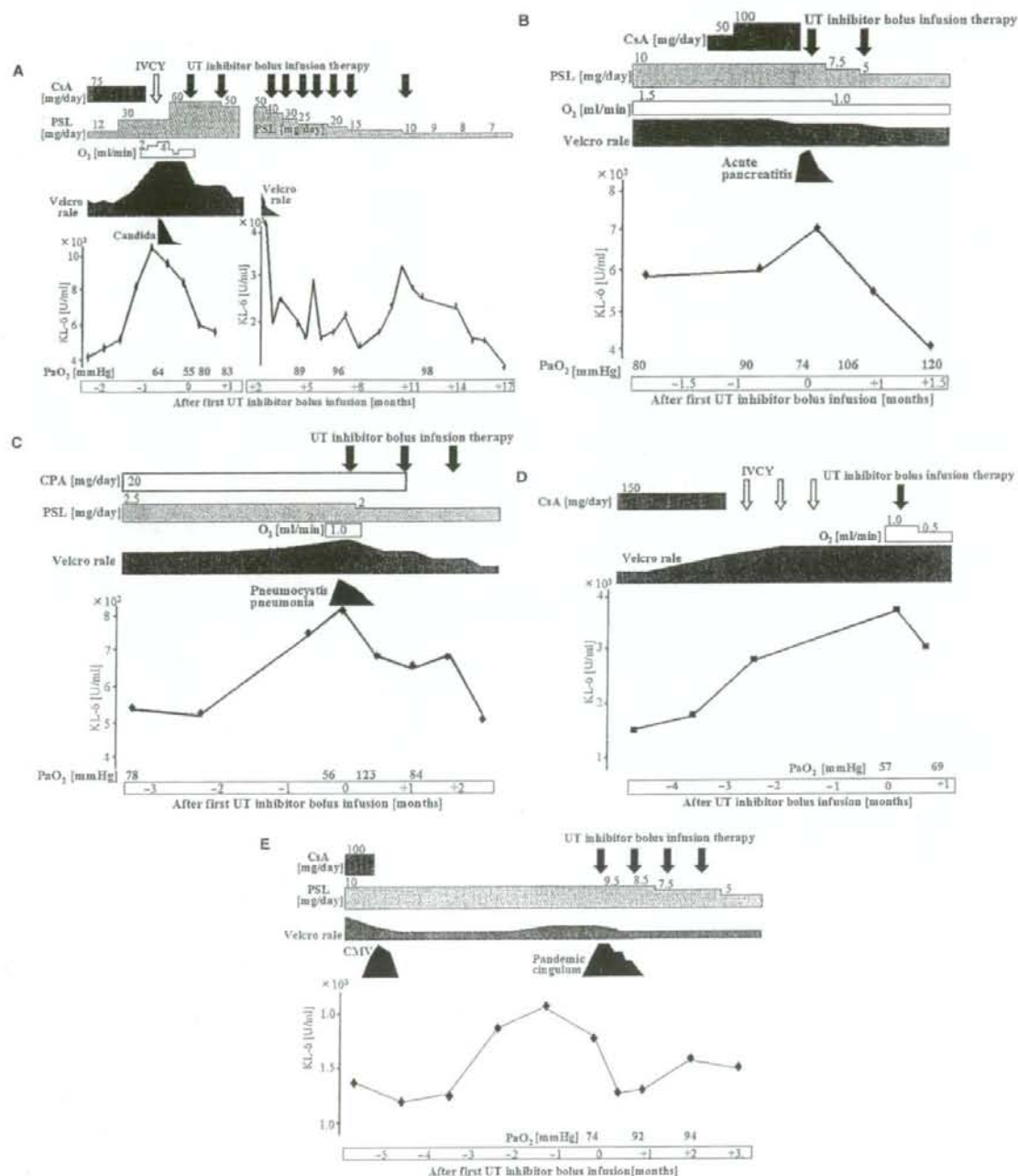
S. Tsujimura *et al.*

FIG. 2. Clinical course of the five patients. (A) Case 1 [19]. (B) Case 2. (C) Case 3. (D) Case 4. (E) Case 5. Open arrows: single IVCY, closed arrows: one course of UT inhibitor bolus infusion therapy. CPA: oral cyclophosphamide; O_2: oxygen inhalation; Candida: fungal pneumonia with *Candida albicans*; CMV: cytomegalovirus viremia; PaO_2: partial pressure of oxygen in arterial blood of Cases 1, 3, 4 and 5 on breathing room air and Case 2 during breathing of 1.0 l/min of oxygen.

organ failure and opportunistic infection. Furthermore, repeated UT inhibitor bolus infusion therapy maintained the state of IP remission and allowed tapering of steroids in Patients 1 [15], 2, 3 and 5.

The pathological changes associated with IP include inflammatory cell accumulation and alveolar damage together with progressive fibrosis and remodelling of the broncheoalveolar tree. These processes are reported to involve various mediators, such as

TABLE 2. Efficacy of a course of UT inhibitor bolus infusion on IP

|	Case 1	Case 2	Case 3	Case 4	Case 5
Combination therapy	PSL 60 mg	PSL 10 mg	PSL 2.5 mg	–	PSL 10 mg
Dyspnoea	Improved	No change	Improved	Improved	Improved
Velcro rales	Decreased	Decreased	Decreased	No change	Decreased
Oxygen supply (l/min)					
Before	2.0	1.5	1.0	1.5	1.0
1 Month after	0	1.0	0	1.0	0
LDH (IU/ml)	208	165	189	212	250
PaO$_2$ (mmHg)	83	120	84	69	92
KL-6 (U/ml)	5392	543	666	2982	1274
Adverse effects	–	–	–	–	–
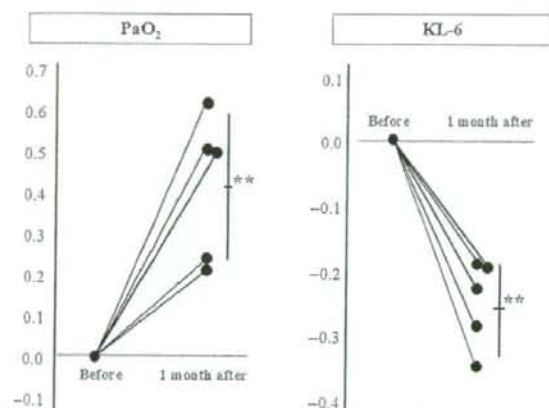
FIG. 3. Improvement of PaO$_2$ and serum KL-6 levels 1 month after one course of UT inhibitor bolus infusion therapy. Data represent the rate of change in each parameter, relative to the respective value measured before UT inhibitor bolus infusion. Data are mean ± s.o. of five measurements. **$P < 0.01$, by paired t-test.
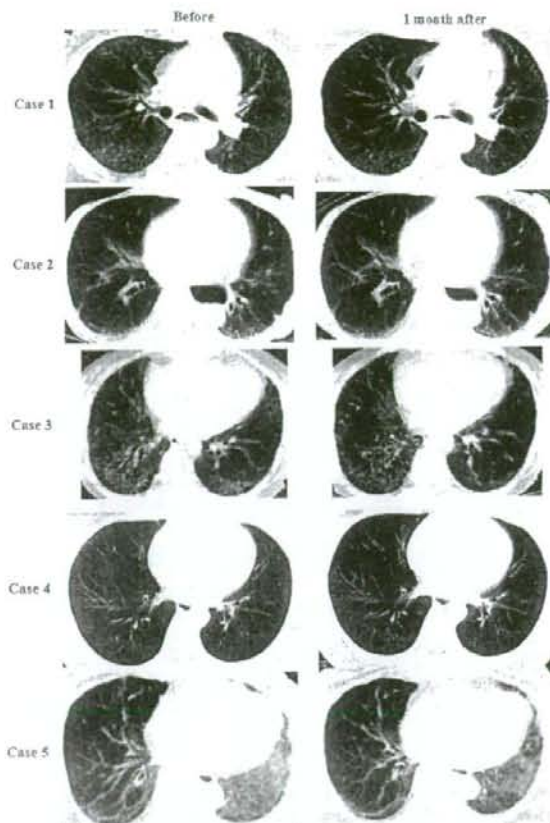

FIG. 4. Chest CT findings before and 1 month after UT inhibitor bolus infusion in the five patients. Paired images from the same axial level are shown sequentially for each patient. See text for further explanation.

oxygen radicals [24], MCP-1 [22], TGF-β1 [23], matrix metalloproteinase (MMP) [25], and adhesion molecules [26]. UT inhibitor is a proteoglycan that increases in response to various injuries in the human body [27, 28]. UT inhibitor is often used for life-threatening inflammation such as acute pancreatitis and acute circulatory collapse. It has anti-inflammatory and organ-protective effects through inhibition of various inflammation-related mediators including proteases [29], cytokines [12], oxygen radicals [13] and adhesion molecules [14]. It has been reported that MCP-1 and TGF-β1 produced by type II alveolar epithelial cells are increased in bronchoalveolar lavage fluid of patients with IP [30, 31]. MCP-1 induces extravascular infiltration of neutrophils, macrophages and Th2 type of lymphocytes. TGF-β1 is also involved in the pathogenesis of IP by up-regulating the proliferation of pulmonary fibroblasts. Actually, in our patients, we observed high levels of MCP-1 and TGF-β1 and the values decreased at 3 h after the initial bolus infusion of UT inhibitor. Others have reported that UT inhibitor provides protection of alveolar epithelial cells by suppressing the release of oxygen radicals from neutrophils, inhibits accumulation of inflammatory cells by suppressing the production of TNF-α and IL-8 and down-regulating the expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells, and could be effective in preventing pulmonary remodelling by suppressing the production of MMP. Thus, these mechanisms of UT inhibitor seem different from those of conventional immunosuppressive, cytotoxic agents. Furthermore, the present cases emphasized the usefulness of UT inhibitor bolus infusion therapy for IP that could not be controlled by conventional therapies.

Although others have suggested that UT inhibitor is effective for paraquat-induced pulmonary fibrosis and idiopathic IP [32, 33], there is no convincing report of the therapeutic efficacy of UT inhibitor for IP with systemic autoimmune diseases. Kamei et al. [33] reported that intravenous infusion of UT inhibitor (1.5×10^5 U × 2/day for 5 days) had no significant effects on ICAM-1, IL-8 and difference of alveolar arterial oxygen partial pressure (AaDO$_2$). One of the reasons for the inadequate effect of UT inhibitor infusion is that the UT inhibitor blood concentration scarcely reaches an effective level because the half-life of UT inhibitor is only 40 min [34]. Others have reported that suppression of production of lipopolysaccharide (LPS)-induced oxygen radicals and cytokines requires more than 30 and 100 U/ml UT inhibitor concentrations, respectively [35, 36]. In addition, concentrations well above 40 U/ml of UT inhibitor are required to suppress MMP production [29]. However, it is almost impossible to obtain these concentrations of UT inhibitor by conventional intravenous infusion through peripheral veins even in the case of 3×10^5 U bolus infusion. Sugiura et al. [37] reported that UT inhibitor concentration was 91.5 U/ml at only 2 min and fell to 52.6 U/ml at 30 min after intravenous bolus infusion of 3×10^5 U of UT inhibitor through peripheral veins. It is notable that we overcame this issue by bolus infusion of UT inhibitor into the superior vena cava using a central venous catheter. The selection of this route ensured sufficient concentrations of UT inhibitor in the pulmonary
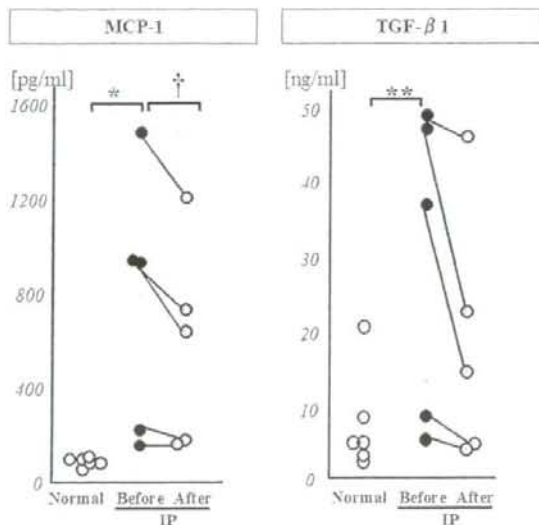
Fig. 5. UT inhibitor bolus infusion reduced the high levels of MCP-1 and TGF-β1 in patients with IP. Arterial blood samples were collected from the brachial artery of normal volunteers and patients with IP before and 3 h after the first UT inhibitor bolus infusion for measurement of serum concentrations of MCP-1 and TGF-β1. *$P < 0.05$, **$P < 0.01$, by non-paired *t*-test. †$P < 0.05$, by paired *t*-test.

circulation (Fig. 1), and probably resulted in reduction of various mediators at the site of pneumonitis.

In conclusion, UT inhibitor bolus infusion therapy may be useful for IP based on its anti-inflammatory and anti-oxidant effects, and on lacking serious adverse effects, such as bone marrow suppression, organ failure and opportunistic infection. However, more cases need to be reported before the establishment of UT inhibitor bolus infusion therapy. We propose that UT inhibitor bolus infusion therapy is potentially useful as an alternative therapy for refractory IP in patients resistant or intolerable to conventional immunosuppressive treatments.

Rheumatology key messages

- UT inhibitor bolus infusion therapy could show anti-inflammatory and anti-oxidant effects without serious adverse effects.
- UT inhibitor bolus infusion therapy could be useful as an alternative therapy for refractory IP.

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