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Edaravone inhibits rheumatoid synovial cell proliferation and migration

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Abstract
Rheumatoid arthritis (RA) is characterized by synovial proliferation and migration which is induced by proinflammatory cytokines or oxidative stress, followed by joint destruction. Edaravone, clinically available free radical scavenger in Japan, is confirmed to be beneficial in the acute stage of cerebral infarction. We aimed to investigate whether edaravone suppressed *in vitro* proliferation and migration of synovial cells (SC) induced by IL-1β. SC proliferation and migration induced by IL-1β were dose-dependently suppressed by edaravone at the clinically available concentration. These data suggest that edaravone has potential effects to suppress SC proliferation and migration, followed by suppression of synovial proliferation in RA. Therefore, edaravone, an antioxidant agent, might be a novel therapeutic agent which develops the new strategy for treatment of RA, and more detailed studies are required to establish the therapeutic effect of edaravone on RA *in vivo*.

Keywords: *Rheumatoid arthritis, synovial cell, proliferation, migration, oxidative stress, free radical*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with immune abnormalities. Synovia in RA is characterized by infiltration of various inflammatory cells and excessive growth and migration of synovial cells (SC), resulting in joint destruction. It is well known that synovial tissues of RA excessively produce proinflammatory cytokines including interleukin-1 (IL-1), which involve SC proliferation and migration by autocrine and/or paracrine mechanisms. On the other hand, several studies revealed that the impaired metabolism of free radicals was present in RA. Excessive free radical production [1] and impairment of antioxidant system [2] was discussed in the inflammatory lesions from RA. Rheumatoid SCs produce many kinds of substances, such as free radicals and nitric oxide. Free radicals may significantly contribute to overproduction of cytokines and hyperproliferation and migration of rheumatoid SCs [3,4]. It was also reported that glycosaminoglycans, endogenous antioxidants, decreased not only plasma TNF-α levels but also limited erosive damage in collagen-induced arthritis in Lewis rats [5]. As the oxidative stress, in general, plays an important role in the inflammatory process, free radicals, as mediators of joint destruction, may also contribute to production of cytokines and SC proliferation and migration in RA.

Edaravone (MCI-186, norphenazone, 3-methyl-1-phenyl-2-pyrazolin-5-one) is a neuroprotective agent that has been confirmed to be useful for the treatment of acute embolic stroke [6] in Japan since, 2001. This compound has potent free radical scavenging and antioxidant actions [7]. Recently, it is also reported that edaravone may be potentially useful in prevention of various diseases occurred by reactive oxygen species, for example, cisplatin-induced nephrotoxicity [8], carbon tetrachloride-induced acute liver injury [9], dextran sulfate sodium-induced colitis [10],

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closed duodenal loop-induced acute pancreatitis in rats [11] and paraquat intoxication in mice [12].

As we had an experience that polyarthralgia improved with a reduction of serum C-reactive protein concentrations soon after the administration of edaravone in a patient with RA incidentally accompanied with cerebral infarction, the aim of this study was undertaken to evaluate whether edaravone was potentially beneficial for the control of RA activity. We demonstrate herein, that edaravone inhibits *in vitro* SC proliferation and migration.

Materials and methods

Cell culture and reagents

Synovial tissues were obtained from three female RA patients (aged 71, 77 and 73 years) with their informed consent in compliance with the declaration of Helsinki for human subjects undergoing total knee replacements. SC were prepared and cultured according to our previous report [13], and then each experiments of edaravone on SC were performed. Edaravone was kindly provided by Mitsubishi Pharma Corporation (Osaka, Japan). Edaravone was dissolved in 1 N sodium hydroxide solution (NaOH), and was adjusted to pH 7.0 with 1 N hydrochloric acid (HCl). In preliminary experiments, we found that the same concentration of vehicle alone (H_2O; NaOH and HCl were mixed and adjusted pH) did not affect the results in our experiment systems (data not shown).

Assessment of cell toxicity and cell proliferation

SC were seeded at a density of 8×10^3 cells per well with Dulbecco's modified Eagle's medium (DMEM; GIBCOBRL/Life Technologies, Grand Island, NY) containing 0.5% fetal calf serum (FCS; GIBCOBRL/Life Technologies) in 96-well culture plate. The toxicity and proliferation assays of SC were performed using CytoTox96 Non-Reactive Cytotoxicity Assay (Promega, Madison, WI) and TetraColor ONE Cell Proliferation Assay System (Seikagaku Kogyo, Tokyo, Japan), respectively. The grade of cytotoxicity was determined using the result of lactate dehydrogenase (LDH) release assay [14,15]. In short, SC were lysed with 1% Triton X-100 in culture medium to obtain a representative maximal LDH release for that particular culture as the positive control with 100% toxicity, prior to each assay. Substrate mixes in assay kit, and cellular supernatant or culture medium alone as the low control were mixed in another 96-well culture plate and measured using a microtiter plate reader at 490 nm of the absorbance. As a consequence, the grade of cytotoxicity was determined using following formula:

$$\text{Cytotoxicity} = \frac{(\text{experimental value} - \text{low control})}{(\text{positive control} - \text{low control})} \times 100$$

As the amount of formazan produced from the tetazolium salt indicates the reactive potential and the viability of cells, in order to measure cell proliferation we used TetraColor ONE Cell Proliferation Assay System. TetraColor ONE including a water-soluble disulfonated tetrazolium salt was added to each culture plate well and incubated for the additional 2 h at 37°C. The absorbance at 450 nm was measured using a microtiter plate reader. As the absorbance which evaluated formazan reaction product was proportional to the number of biologically active cells in this assay kit [16,17]. The relative proliferation index of SC was estimated by the ratio of the absorbance to the control condition. Tenth and twelveth wells were served as each condition of experiment for cell toxicity assay and cell proliferation assay, respectively. Results are reported as mean ± standard deviation (SD). Each experiment was repeated, at least, three times for respective patients.

Assessment of cell migration

The migration assays of SC [18] stimulated by IL-1β were performed using Chemotaxicell culture chamber (Chemotaxicell, Kurabo Industries Ltd, Osaka, Japan). SC was seeded at a density of 5×10^4 cells per well in upper chamber, and chemoattractant 10 ng/ml IL-1β was placed in lower chamber. The number of SC that had migrated from the upper surface to the lower surface of the filter membrane was determined using confocal laser microscopy. Results are reported as mean ± SD ($n = 10$). The similar experiments were performed at least three times for respective patients.

Statistical analysis

Statistical analysis of data was performed using a software program for use with personal computers (JMP, version 3.1; SAS Institute, Cary, NC). Comparisons for two groups were performed using the Tukey–Kramer test. Statistical differences among three groups or more were determined by analysis of variance (ANOVA). P values < 0.05 were considered statistically significant.

Results

Effect of edaravone on cell toxicity

Edaravone did not disclose any cytotoxic effect on SC measured by LDH release assay from 24 to 72 h:

9.8 ± 0.6% at 24 h (control 10.5 ± 0.6), 10.3 ± 0.5 at 48 h (9.5 ± 0.5) and 11.1 ± 0.6 at 72 h (10.4 ± 0.6).

Effect of edaravone on SC proliferation

The time course of SC proliferation affected by edaravone was shown in Figure 1. The relative proliferation index of SC was analyzed at 0, 24, 48 and 72 h. One micromole edaravone inhibited SC proliferation in 24 h and this effect of edaravone continued for, at least, 72 h. Additionally, it was shown that edaravone did not decrease SC number after incubation. We further examined the dose-dependent effect of edaravone on SC proliferation 48 h after incubation with IL-1β at varying concentrations (Figure 2). Edaravone from 1 to 10 μM dose-dependently inhibited SC proliferation not only at baseline (without IL-1β) condition, but also in stimulated condition by IL-1β at any concentrations from 0.01 to 1 ng/ml. In addition to the effects of single administration of edaravone, we also examined the effects of once-daily treatment for 5 days of edaravone on SC proliferation. As shown in Figure 3, on once-daily treatment with 1 or 10 μM edaravone for 5 days, SC proliferation stimulated by IL-1β of 1 ng/ml was remarkably suppressed by 45 or 66%, respectively (relative proliferation index; 100.0 ± 7.6% at control group of 0 ng/ml IL-1β and 0 μM edaravone; 195.0 ± 15.3% at group of 1 ng/ml IL-1β and 0 μM edaravone, 152.3 ± 12.5% at group of 1 ng/ml IL-1β and 1 μM edaravone and 132.5 ± 14.4% at group of 1 ng/ml IL-1β and 10 μM edaravone).

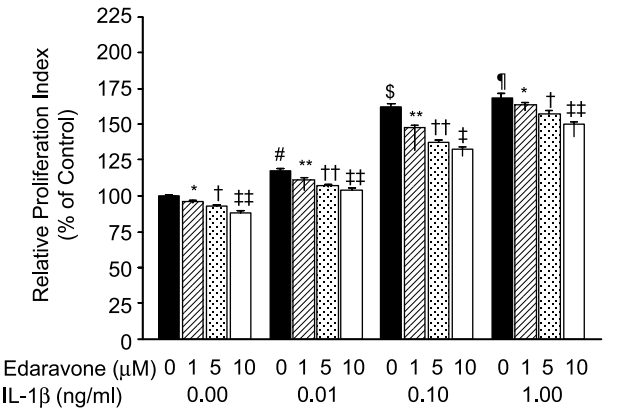

Figure 2. Dose-dependent effects of edaravone on synovial cells (SC) proliferation. SC proliferation was assayed 48 h after stimulation with various concentrations of IL-1β (0, 0.01, 0.1 and 1 ng/ml) and edaravoe (0, 1, 5, and 10 μM) using the TetraColor ONE Cell Proliferation Assay System. Each column represents mean ± SD of data from 12 wells. IL-1β induces synovial cell proliferation dose-dependently ($P < 0.0001$, one-way ANOVA). Edaravone dose-dependently inhibited IL-1β-induced cell proliferation at each concentration of IL-1β ($P < 0.0001$, one-way ANOVA). #, $ and ¶ indicate statistically significant ($P < 0.01$) for IL-1β at 0, 0.01 and 0.1 ng/ml, respectively. ⋆ and ⋆⋆ indicate statistically significant ($P < 0.001$ and $P < 0.0001$) for 0 μM edaravone, † and †† for 1 μM edaravone, and ‡ and ‡‡ for 5 μM edaravone in each IL-1β concentration, respectively.

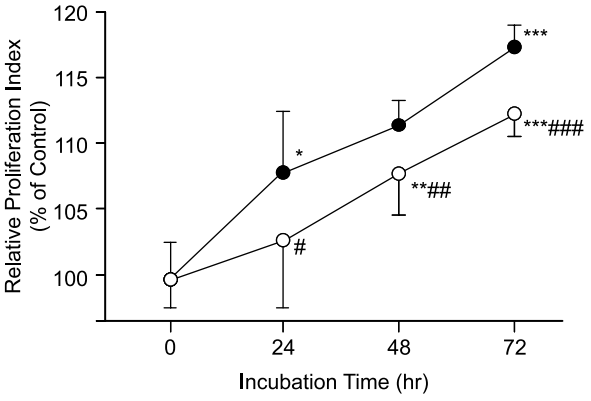

Figure 1. The time course of synovial cells (SC) proliferation. The effects of edaravone on SC proliferation at varying times. SC proliferation was assayed from 0 to 72 h after incubation with 0 μM edaravoe (closed circle) or 1 μM edaravoe (open circle) using the TetraColor ONE Cell Proliferation Assay System. The results represent mean ± SD from 10 wells. The number of SC with both 0 and 1 μM edaravoe increased from 0 to 72 h ($P < 0.0001$, one-way ANOVA). Abbreviations; ⋆, $P < 0.0001$ vs. 0 h; ⋆⋆, $P < 0.0001$ vs. 24 h; ⋆⋆⋆, $P < 0.0001$ vs. 48 h in each edaravone concentration, respectively; #, $P < 0.001$ vs. 0 μM edaravone at 24 h; ##, $P < 0.01$ vs. 0 μM edaravone at 48 h; ###, $P < 0.01$ vs. 0 μM edaravone at 72 h.

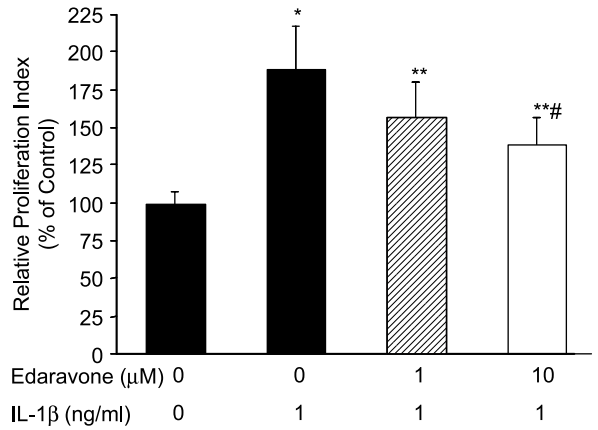

Figure 3. The effects of edaravone for 5 days on synovial cells (SC) proliferation. SC proliferation was assayed 5 days after stimulation with various concentrations of IL-1β (0 and 1 ng/ml) and edaravoe (0,1 and 10 μM) using the TetraColor ONE Cell Proliferation Assay System. Each column represents mean ± SD of data from 12 wells. Edaravone dose-dependently inhibited IL-1β-induced cell proliferation ($P < 0.0001$, one-way ANOVA). ⋆ indicate statistically significant ($P < 0.0001$) for IL-1β at 0 ng/ml, ⋆⋆ indicate statistically significant ($P < 0.0001$) for IL-1β at 1 ng/ml and 0 μM edaravone, # indicate statistically significant ($P < 0.01$) for IL-1β at 1 ng/ml and 1 μM edaravone.

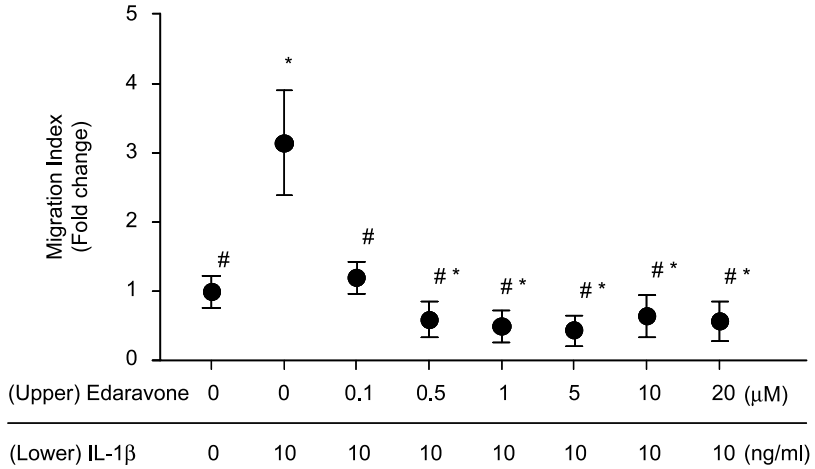

Figure 4. The effects of edaravone on synovial cells (SC) migration. SC migration was evaluated using Chemotaxicell culture chamber ($n = 10$). Eighteen hours after stimulation of SC with 10 ng/ml IL-1β, SC migration was induced. IL-1β-induced SC migration was inhibited by edaravone in dose-dependent manner. Abbreviations; ⋆, $P < 0.05$ vs. 0 μM edaravone and 0 ng/ml IL-1β; #, $P < 0.05$ vs. 0 μM edaravone and 10 ng/ml IL-1β.

Effect of edaravone on SC migration induced by IL-1β

Eighteen hours after stimulation of SC with 10 ng/ml IL-1β, SC migration was induced (Figure 4). IL-1β-induced SC migration was inhibited by edaravone at the concentrations from 0.1 to 20 μM.

Discussion

Pathogenesis of RA is characterized by synovial proliferation, followed by pannus formation and destruction of bone and cartilage. Therefore, the suppression of SC proliferation and migration is one of the major therapeutic strategies for the treatment of RA. In this study, we confirmed that edaravone was effective on suppression of SC proliferation and migration at the available concentrations (< 6 μM), which corresponded to the concentrations in the sera of patients receiving intravenous administration of edaravone for acute embolic strokes. There was 10 or 29% suppression of SC proliferation in the single treatment of SC with 1 or 10 μM edaravone for 48 h, respectively (Figure 2). Moreover, on once-daily treatment with 1 or 10 μM edaravone for 5 days, SC proliferation stimulated by IL-1β of 1 ng/ml was remarkable suppressed by 45 or 66%, respectively (Figure 3). Consequently, the administration of edaravone is likely to inhibit synovial proliferation and to be a novel therapeutic approach for joint destruction in RA.

We do not know the precise mechanism(s) by which edaravone inhibits SC proliferation and migration. There has been reportedly discussed on the implication of antioxidant system in the treatment for free radical pathogenesis in rheumatoid inflammation. Several drugs commonly used for RA such as gold salts, penicillamine, methotrexate and non-steroidal anti-inflammatory drugs, have antioxidant action as one of multiple anti-rheumatic mechanisms, which might contribute to suppress the joint destruction. Recently, it was reported that administration of drug plants [19] and intraarticular administration of superoxide dismutase [20] were useful for the treatment of RA by antioxidant action. Prospective cohort study reported that intake of certain antioxidant micronutrients [21] and abstinence from smoking [22] also protected against the development of RA. Our results that edaravone, antioxidant suppressed SC proliferation and migration in this study were mechanically consistent with these reported aspects of pathogenesis in RA. It is also worthy of note that edaravone was comprehended enough to suppress the RA activity in our RA patient who received intravenous administration of edaravone for complicated acute embolic strokes (unpublished observation).

The biological agents targeting tumor necrosis factor α, infliximab [23] and etanercept [24], have been recently confirmed to be markedly effective for control of RA, however, sometimes cause serious illness incidentally. There has been reported some adverse reactions of edaravone for the treatment of acute brain infarction [6], which are, however, neither frequent nor serious in comparison with disease-modified anti-rheumatic drugs we commonly use. Therefore, our results suggest that edaravone is clinically useful in the treatment of RA.

In conclusion, the previous observations and our preliminary *in vitro* observations of edaravone on SC suggest that edaravone suppresses the disease activity of RA, although the precise mechanism(s) of edaravone remain unclear. We also have the preliminary *in vivo* effect of edaravone on the suppression of joint inflammation in collagen-induced mice model (unpublished observation). Therefore, edaravone, an

antioxidant agent, might be a novel therapeutic agent which develops the new strategy for treatment of RA, and more detailed studies are required to establish the therapeutic effect, the possible mechanisms, and implication of edaravone on RA *in vivo*.

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