Enhanced Anti-Inflammatory Effects of Cu, Zn-Superoxide Dismutase Delivered by Genetically Modified Skin Fibroblasts *In Vitro* and *In Vivo*

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Purpose. The purpose of this work was to evaluate the anti-inflammatory effects of secretable human Cu, Zn-superoxide dismutase (hSOD) delivered by genetically modified skin fibroblasts *in vitro* and *in vivo*.
Methods. Rat skin fibroblasts were transfected with pRc/CMV-ILSOD including secretable SOD-coding cDNA. The effects of host and transformants on oxidative stress *in vitro* models using the xanthine/xanthine oxidase (X/XO) system were examined to study the paracrine SOD action. The anti-inflammatory effects by transplantation of host and transformants were evaluated in an acute inflammation model, carrageenin-induced paw edema, in rats.
Results. The transformants (ILSOD cells) secreted SOD protein into the extracellular space, and the extracellular SOD activity in ILSOD cells cultures was significantly increased compared with that in host cell cultures. ILSOD cells diminished the cytotoxic activity by X/XO in a paracrine fashion. These protective effects of ILSOD cells against X/XO-induced cytotoxicity correlated well with the decrease in lipid peroxidation in the damaged cells. The *in vivo* study showed that transplantation of ILSOD cell suspensions into the hind paw in rats inhibited carrageenin-induced paw edema for at least 7 days, and the degree and the durability of these inhibitory effects were dependent on the number of ILSOD cells transplanted. These inhibitory effects of ILSOD cell suspensions were reduced by co-administration of antiserum for hSOD. Furthermore, the healing of paw edema caused by carrageenin was markedly enhanced by transplantation of ILSOD cells into the edemics hind paw.
Conclusions. The findings suggested that genetically modified skin fibroblasts are a suitable delivery system for obtaining an efficient and continuous supply of SOD to the target site, and this strategy may be a useful drug delivery system for therapeutic proteins.

KEY WORDS: gene therapy; SOD; cell transplantation; xanthine/xanthine oxidase; carrageenin; paw edema.

INTRODUCTION

Human Cu, Zn-superoxide dismutase (hSOD), which is a cytoplasmic enzyme, cleaves the superoxide radical to molecular oxygen and hydrogen peroxide, thus helping to protect cells against the toxic byproducts of aerobic metabolism (1) and making SOD clinically useful as a nonsteroidal anti-inflammatory protein (2). Indeed, its efficacy has been reported for treatments of several diseases including inflammation (3), ischemic damage (4) and others (5). However, the short half-life in blood of SOD due to its rapid removal via filtration by the kidney limits the clinical usefulness of this enzyme (6). Therefore, a new delivery system for SOD is necessary to obtain an efficient and continuous supply to the target sites. Genetically modified autologous cells from patients can produce the desired protein in target sites, and thus can be used as effective carriers for these molecules. Skin fibroblasts are attractive candidates because they offer several advantages, such as easy access, ease of culture *in vitro*, efficient transfection with plasmid DNA, and convenient reimplantation. Several studies have shown the feasibility of this approach by transplanting genetically modified fibroblast cell lines into laboratory animals and detecting the expression of the heterologous proteins (7,8). Previously, we constructed a plasmid vector which expressed a secretable hSOD and demonstrated that lung epitherial-like cells transformed with this vector could produce secretable hSOD and showed reduced oxidative stress *in vitro* (9).

In the present study, it was confirmed that genetically modified skin fibroblast cells carrying the secretable hSOD expression plasmid secreted hSOD protein into the extracellular space and that *in vitro*, these transformants reduced the enzymatically induced oxidative stress in a paracrine fashion. Furthermore, topical transplantation of these cells showed enhanced anti-inflammatory effects of hSOD, and these effects lasted longer than those with administration of recombinant hSOD.

MATERIALS AND METHODS

Materials

Recombinant human Cu, Zn-SOD (rhSOD) (3200 U/mg protein) was supplied by Asahi Chemical Industries (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, xanthine oxidase (XO), penicillin-streptomycin mixture, and fetal bovine serum (FBS) and carrageenin (Picnin-A®) were purchased from Flow Laboratories, Life Technologies, Boehringer Mannheim GmbH (Mannheim, Germany), Bio Whittaker, and Zushi Chemicals (Tokyo, Japan), respectively. Trypsin solution, xanthine (X), geneticin disulfate (G418) and trypan blue were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade.

Cell Culture

The rat skin fibroblast cell line ATCC CRL 1213 (FR cells) was obtained from the American Type Culture Collection and was grown in DMEM containing 10% FBS and a penicillin-streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2. Culture medium for transfected FR cells was supplemented with G418 (final conc. 400 μg/ml) to maintain selection pressure, and with $CuSO_4$ and $ZnSO_4$.

Transfection

The expression plasmid used in this study, pRc/CMV-ILSOD, was constructed previously (9) and contained the cytomegalovirus (CMV) promoter, the neomycin resistance gene (neo^r), the β-lactamase gene and an IL-2 signal peptide/11-amino acids connecting peptides/hSOD fusion protein-coding

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cDNA (ILSOD cDNA). Transfection of FR cells with pRc/CMV-ILSOD was accomplished using Lipofectin® (GIBCO/BRL) according to the previous report (10). For the isolation of single transfectants, clonal rings were used, and at least 30 single clones were further expanded under continued G418 selection. After determination of the extracellular SOD activity in each clone, that with the highest extracellular SOD activity was selected. This clone was used as the ILSOD cell line in the experiments as described below.

Western Blot Analysis for hSOD and SOD Activity Assay

Approximately 90% confluent cells in dishes 100 mm in diameter were washed with phosphate-buffered saline (PBS), and overlaid with 10 ml of serum-free DMEM (without phenol red). After incubation for 24 h, 5 ml of media was removed and dialyzed using wet cellulose dialysis tubing (M.W. cutoff 10000, SPECTRAPOR®, Spectrum Medical Industries, LA). This dialyzed sample was lyophilized and then dissolved in distilled water (50 μl). hSOD protein was detected by Western blotting analysis using anti-hSOD whole serum (The Binding Site, Birmingham, UK) according to the method described in the previous paper (11). For measurement of SOD activity in media, cells (5×10^5 cells) were seeded in 100-mm dishes and incubated in DMEM containing 10% FBS for 18 hours. The media was replaced with serum-free DMEM (without phenol red) and incubated for 24 h, then SOD activity in media was determined by the method of Oyanagui *et al.* (12) using rhSOD as a standard. The numbers of cells in cultures were also estimated by the trypan blue exclusion method.

Xanthine/Xanthine Oxidase (X/XO)-Induced Cytotoxicity *In Vitro*

X/XO-induced cytotoxicity was evaluated as described in the previous paper (9) as follows. Cells (2×10^5 cells) were seeded in 35-mm dishes and incubated in DMEM with 10% FBS and antibiotics. After incubation for 24 h, media in cultures of host or ILSOD cells were replaced with 2 ml of conditioned medium obtained from host or ILSOD cell cultures as described below. XO (final conc. 5 mU/ml) was then added to each culture and incubated at 37°C for 24 h. Cell survival and lipid peroxidation were determined by the trypan blue exclusion method and with a lipid peroxidation kit (Wako Pure Chemicals, Osaka, Japan), respectively. Lipid peroxidation was expressed as malondialdehyde (MDA) equivalents per cell. The preparation of conditioned medium from each cell culture was performed as follows. Cells (5×10^5 cells) were seeded in 100 mm dishes and incubated in DMEM containing 10% FBS for 3 days. Media were then replaced with serum-free DMEM containing X (final conc.; 0.5 mM), $CuSO_4$ and $ZnSO_4$. After incubation for 24 h, the media were collected in test tubes as incubated medium. The numbers of cells in these cultures were estimated, and incubated media were then adjusted to 10^4, 10^5, and 10^6 cells/ml with serum-free DMEM containing X (final conc.; 0.5 mM), $CuSO_4$ and $ZnSO_4$. These adjusted media were used as the conditioned media.

Cell Transplantation and Carrageenin-Induced Paw Edema in Rats

Under light ether anesthesia, carrageenin in saline (1% or 2%, 100 μl) was injected s.c. into the hind paw pads of male Wistar rats (157 ± 2 g). The paw volume was measured by immersion in the cuvette of a water plethysmometer (13) immediately before and at the indicated times after injection. To evaluate anti-inflammatory effects of transplantation of cells, control (100 μl of PBS only) or cell suspensions (3×10^5 or 3×10^6 cells in 100 μl of PBS, respectively) were injected s.c. into the hind paw pad on 1, 2, 3 or 7 days before, or 1 or 4 h after injection of carrageenin.

Statistics

Data represent means ± SE. Statistical analysis was performed by Student's *t*-test or ANOVA. A level of $p < 0.05$ was considered significant.

RESULTS

Transfection with pRc/CMV-ILSOD and Characterization of ILSOD Cells

As shown in Fig. 1A, Western blots showed two hSOD-related bands in the medium from ILSOD cell cultures (lane 3), whereas no band was observed in that from host cell cultures (lane 4) or in cell-free medium (lane 2). Lanes 1 and 5 showed the rhSOD protein as a standard. In addition, the extracellular SOD activity in ILSOD cell cultures was significantly increased (about 1.8-fold) relative to that in host cell cultures (Fig. 1B). However, the cellular SOD activity in ILSOD cells was not different from that in host cells (Fig. 1C).

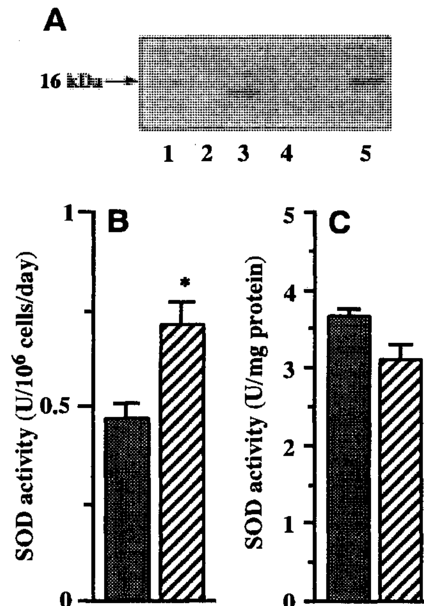

Fig. 1. Confirmation of expression of ILSOD cDNA in FR cells. (A) Immunoblot analysis using anti-hSOD antiserum. Each sample was applied onto SDS-PAGE for immunoblot analysis. Lanes: 1 and 5, rhSOD (1 and 10 ng, respectively); 2, cell-free medium; 3, conditioned medium from ILSOD cells; 4, conditioned medium from host cells. (B) Extracellular SOD activity. (C) Intracellular SOD activity. ■, Host cells; ▨, ILSOD cells. Bars represent means ± SE (n = 3 − 10). *, $p < 0.05$.

Effects of Conditioned Medium on Oxidative Stress *In Vitro*

To determine if genetically modified skin fibroblasts expressing secretable SOD could be utilized as a delivery system for SOD to tissues damaged by oxidative condition, we investigated the effects of ILSOD cells on oxidative stress using X/XO system *in vitro*. As a model of paracrine activity of secretable SOD, we examined the protective effects of the conditioned media from host or ILSOD cell cultures on adjacent host cells exposed to X/XO (Fig. 2). The decrease in cell survival in the adjacent cells exposed to X/XO (0.5 mM/5mU/ml) was inhibited depending on concentrations of the conditioned medium. However, at each concentration (10^4, 10^5 and 10^6 cells/ml), the conditioned media from ILSOD cell cultures showed a significantly greater inhibitory effect on the cytotoxicity of X/XO on adjacent cells in comparison with those from host cell cultures. Furthermore, the changes in MDA level as an indicator of lipid peroxidation in adjacent cells exposed to X/XO paralleled those of cell survival, and 10^6 cells/ml of conditioned medium from ILSOD cell cultures completely inhibited both the decrease in cell survival and the increase in MDA level in adjacent cells exposed to X/XO. These findings indicate that ILSOD cells can protect adjacent cells from extracellular oxidative stress in paracrine fashion.

Effects of Transplantation of ILSOD Cells on Carrageenin-Induced Paw Edema in Rats

Subcutaneous injection of carrageenin, a potent inducer of acute local inflammation, increases the permeability of vascular endothelial cells and induces paw edema in rats (3). As shown in Fig. 3, we examined the effects of administration of rhSOD and transplantation of ILSOD cells on carrageenin-induced paw edema in rats. The intravenous (i.v.) administration of rhSOD (3000 U/kg) 30 min before carrageenin injection partially inhibited paw edema in comparison with that of control; however, i.v. administration of rhSOD (3000 U/kg) 1 day before carrageenin injection had no such effect on paw edema (Fig. 3A). Similar findings were observed with subcutaneous (s.c.) administration of rhSOD (300 U/paw) to the paw 1 day before injection of carrageenin in comparison with that of control (Fig. 3B). These observations indicated that the rapid disappearance of hSOD from both blood and target tissue is contributed to the poor response to edema. As shown in Fig. 3C–F, the paw volume following transplantation of host or ILSOD cells or injection of only PBS as a control increased with time until 4 h after injection of carrageenin. The transplantation of ILSOD cells (3×10^6 cells) significantly suppressed the increase in paw volume in comparison with that of control or host cells until 7 days after transplantation. However, transplantation of 3×10^5 cells inhibited paw edema for only 3 days. Following transplantation of ILSOD cells (3×10^6 cells), the increase in paw volume was less than that after transplantation of 3×10^5 cells. On the other hand, injection of control or host cells (3×10^6 cells) had no suppressive effect on carrageenin-induced paw edema.

As shown in Fig. 4, the inhibitory effects by transplantation of an other transformant type, CMV-SOD cells, i. c., the increase in intracellular SOD activity but not in extracellular SOD activity (10), were not observed in comparison with that of control (PBS only). Furthermore, the suppression of carrageenin-induced paw edema after transplantation of ILSOD cells (3×10^6 cells) was inhibited by co-administration of antiserum for hSOD (Fig. 5). These findings indicated that the suppression of carrageenin-induced paw edema by transplantation of ILSOD cells was due to the release of secretable SOD protein from ILSOD cells.

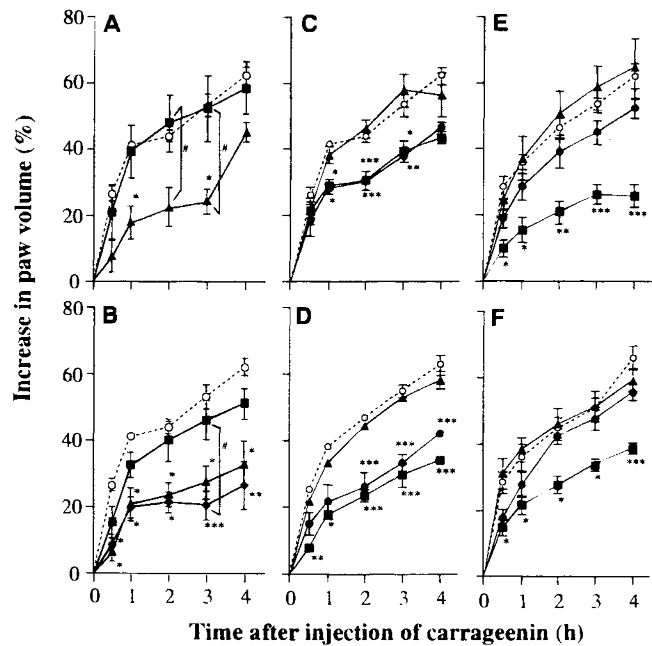

Fig. 3. Effects of administration of rhSOD and transplantation of ILSOD cells on carrageenin-induced paw edema in rats. (A) Effects of i.v. administration of rhSOD. Carrageenin (2%) was s.c. injected into the paw 30 min (▲) or 1 day (■) after i.v. injection of rhSOD (3000 U/kg), and 1 day after i.v. injection of control (saline, ○). (B) Effects of s.c. administration of rhSOD. Carrageenin (2%) was s.c. injected into the paw 30 min after s.c. injection of 3 U/paw (▲) and 30 U/paw (◆) of rhSOD, and 1 day after s.c. injection of control (saline, ○) and 300 U/paw of rhSOD (■). (C–F) Effects of transplantation of ILSOD cells. Carrageenin solution (2%) was s.c. injected into the paw 1 day (C), 2 days (D), 3 days (E), and 7 days (F) after injection of control (PBS, ○), host cells (3×10^6 cells, ▲), ILSOD cells (3×10^5 cells, ●) or ILSOD cells (3×10^6 cells, ■). Points represent means ± SE (n = 4). *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.005$ compared with control, and # $p < 0.05$.

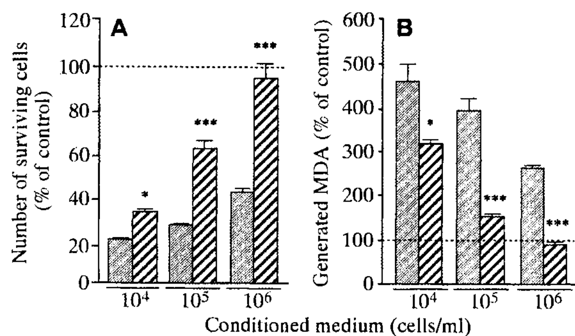

Fig. 2. Protective effects of conditioned medium on X/XO-induced cytotoxicity in FR cells. (A) Surviving cells. (B) Lipid peroxidation. ▒, conditioned medium from host cells; ▨, conditioned medium from ILSOD cells. Bars represent means ± SE (n = 4). *, $p < 0.05$ and ***, $p < 0.005$.

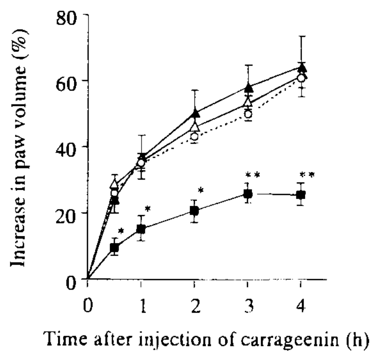

Fig. 4. Effects of transplantation of CMV-SOD cells on carrageenin-induced paw edema in rats. Carrageenin solution (2%) was s.c. injected into the paw 3 days after transplantation of host cells (△), ILSOD cells (■), or CMV-SOD cells (▲). Of each cells, 3×10^6 was transplanted. PBS was administered as a control (○). Points represent means ± SE (n = 4). *; $p < 0.05$ and **; $p < 0.01$ compared with other groups.

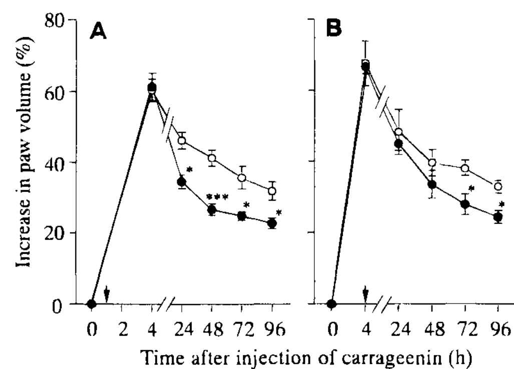

Fig. 6. Inhibition of paw edema by transplantation of ILSOD cells after injection of carrageenin solution in rats. Host (○) or ILSOD (●) cells (3×10^6 cells, respectively) were transplanted into the paw 1 h (A) and 4 h (B) after injection of carrageenin solution (1%). Points represent means ± SE (n = 4). *, $p < 0.05$ and ***, $p < 0.005$ compared with the transplantation of host cells. The times of transplantation of cells are indicated by the filled arrows.

The paw edema caused by carrageenin injection was significantly improved by transplantation of ILSOD cells (3×10^6 cells) 1 h or 4 h after injection in comparison with host cells (Fig. 6). Also, the healing pattern after transplantation of host cells did not change in comparison with that of controls and was not influenced by the transplantation schedule. In contrast, although the significant enhancement of healing by transplantation of ILSOD cells 1 h after carrageenin injection was observed from 24 h to 96 h, transplantation at 4 h had no effect until 72 h. The paw volume at 96 h was not significantly different between animals injected with ILSOD cells at 1 h or 4 h after administration of carrageenin but was significantly reduced in comparison with that in animals administered host cells. These results indicate that the transplantation of ILSOD cells into inflamed sites can enhance healing and that the transplantation time of ILSOD cells is important in obtaining the anti-inflammatory activity.

DISCUSSION

Several attempts have been made to improve the pharmacokinetic characteristics of SOD by use of liposomes and modification with polyethylene glycol, leading to a longer plasma half-life and slower release; but an efficient dismutation of the superoxide could not be obtained by a single dose of these preparations (14–16). However, in this study, we showed that genetically modified skin fibroblasts which express secretable SOD were a suitable topical and continuous delivery system of SOD to inflamed tissue.

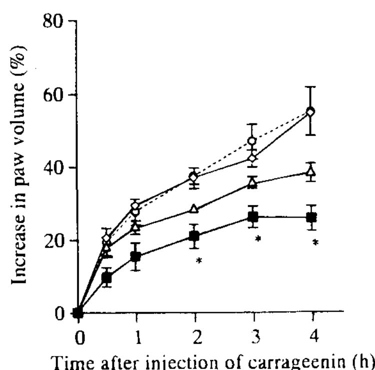

Fig. 5. Effects of antiserum for hSOD on suppression of carrageenin-induced paw edema by transplantation of ILSOD cells in rats. Carrageenin solution (2%) was injected s.c. into the paw 3 days after s.c. injection of ILSOD cells alone (3×10^6 cells, ■), antiserum for hSOD alone (○), or coadministration (◇, △). The volume of antiserum injected was 10 μl (○, ◇) or 1 μl (△). Points represent means ± SE (n = 4). *, $p < 0.05$ compared with injection of antiserum alone.

On Western blot analysis, a band larger than standard rhSOD was observed in the medium from ILSOD cell cultures (Fig. 1A). This band may have been derived from hSOD fused with the N-terminal sequence of IL-2 (11 amino acids) as described in the previous paper (9). Sasada *et al.* reported that the level of expression and secretion of the constructed gene-derived products depended on the length of the N-terminal peptide derived from IL-2 (17). In addition, since polyclonal rabbit antiserum for hSOD was used in Western blot analysis, a band of lower molecular weight than standard rhSOD was observed in the medium from ILSOD cells culture. hSOD exists as a homodimer in the cytosol, which is the active form. It was reported that transgenic mice and transformant with hSOD gene expressed the biologically active chimeric dimers of SOD (18,19). Hallewell *et al.* (16) noted that genetically engineered polymers of hSOD comprised of two subunits linked by a 19-amino acid sequence, constructed the biologically active dimer. Thus, we speculated that the lower molecular weight band was due to cross-reactivity with endogenous rat SOD derived from heterodimerization with secretable SOD in the intracellular space. Therefore, the increase of SOD activity in medium from ILSOD cell cultures (Fig. 1C) may have been due to both secretable hSOD and endogenous rat SOD.

The results described in Fig. 2 indicated that the transplanted ILSOD cells were able to protect, in a paracrine fashion, adjacent cells and/or tissues. Since it was reported that oxygen-derived free radicals attack cell membranes and initiate lipid peroxidation (20), the increased resistance to X/XO-induced cytotoxicity by conditioned medium from ILSOD cells may result from the action of secreted SOD. Furthermore, in an autocrine manner, ILSOD cells were more resistant to X/XO-induced cytotoxicity than host cells (data not shown). These indicated that the anti-inflammatory effects obtained by the

transplantation of ILSOD cells may be synergistically enhanced because a greater number of the transplanted cells in the inflamed sites remained viable due to their increased resistance toward the extracellular oxidative stress.

To determine the clinical efficacy of these strategies, we applied these regimens to an animal model *in vivo*; we used carrageenin-induced paw edema in rats as an inflammation model. It was suggested that SOD asserts its anti-inflammatory effect by acting upon the second phase caused by PGs in the swelling of carrageenin-induced paw edema (21). This animal model has been used extensively to determine the activity of non-steroidal anti-inflammatory drugs. Oyanagui (21) suggested that the repeated i.v. administration of bovine SOD was necessary for the maximum inhibition of carrageenin-induced paw edema. Our observations (Fig. 3A and B) also indicated that rhSOD was rapidly cleared from both blood and target tissue. In contrast, carrageenin-induced paw edema was markedly inhibited by prior transplantation of ILSOD cell suspensions (Fig. 3C–F). These inhibitory patterns were essentially in agreement with the results of a previous study (21,22). The inhibitory effects and its durability depended on the number of transplanted ILSOD cells, and the transplantation of ILSOD cell (3×10^6 cells) suspension supported these inhibitory effects for at least 7 days. The durability of these effects by a single transplantation was markedly improved in comparison with the effects of administration of rhSOD or conventional NSAIDs of which the elimination half-lives are only a few minutes or hours, respectively. The results described in Fig. 4 and Fig. 5 indicated that the inhibitory effects by transplantation of ILSOD cells on carrageenin-induced paw edema were primarily due to the secretable SOD protein produced and released by ILSOD cells. Following transplantation of 3×10^6 cells, these anti-inflammatory effects became most prominent at day 3, but the effects were diminishing by day 7 (Fig. 3). Also, in the preliminary examination, we observed that the expression of secretable SOD mRNA on day 7 was about half of those on day 1 at the sites adjacent to the region of transplantation of ILSOD cells (3×10^6 cells) (data not shown). Such a decline by day 7 of the anti-inflammatory effect could be due to the terminal differentiation of the cells carrying the ILSOD cDNA forced by the activation of immune reactions in rats. Hughes *et al.* (23) demonstrated that the transfected fibroblasts enclosed in alginate-poly-L-lysine alginate microcapsules remained viable for over 5 months. Therefore, these problems can possibly be overcome by the improvement of implantation techniques to maintain the transplanted cells for as long as possible.

In the present study, we demonstrated that the secretable SOD from genetically modified skin fibroblasts has potent anti-inflammatory effects. These findings may be useful in treating pathophysiological conditions such as adult respiratory distress syndrome (24) and reumatoid arthritis, which are related to superoxide release, and for hereditary diseases such as familial amyotrophic lateral screosis (25), caused by mutations in the SOD gene. We believe that this approach utilizing recombinant cell-mediated gene therapy might also be used to deliver other therapeutic proteins which can act in a paracrine mode.

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