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Immunosuppressants enhance superoxide radical/nitric oxide-dependent dexamethasone suppression of ischemic paw edema in mice

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Abstract

A possible new common action of immunosuppressants, besides suppression of the genes for cytokines like interleukin-2, was investigated in in vivo models. Dexamethasone (0.1 mg/kg, s.c.) failed to suppress ischemic paw edema in mice 1 h after its injection, but maximal suppression was achieved at 3 h (20%) whereafter the suppression decreased at 6 and 18 h (11% and 10%). Pretreatment with oral FK506 (chemical name is recently donated as tacrolimus, 0.1 mg/kg) resulted in 38%, 52%, 23% and 17% suppression at 1, 3, 6 and 18 h, respectively. Cyclosporin A (1 mg/kg), rapamycin (0.1 mg/kg) and deoxyspergualin (1 mg/kg) showed a similar pattern of suppressions after dexamethasone. Transforming growth factor-β1 (TGF-β1, 0.3 μg/kg, i.p.) maintained the suppression elicited by an immunosuppressant (42–58%) at 6 h after dexamethasone, whereas transforming growth factor-β1 and/or an immunosuppressant were not suppressive. Suppression, irrespective of the agent that elicited it, was blocked by nitric oxide (NO) synthase inhibitor, anti-oxidant enzymes and cycloheximide. Endogenous nitric oxide or oxyradicals are essential for the action of dexamethasone in vivo. The four immunosuppressants bound to specific heat-hock proteins (hsp) in the glucocorticoid receptor complex and might enhance the synthesis of anti-inflammatory protein(s). © 1998 Elsevier Science B.V.

Keywords: Dexamethasone; FK506; Nitric oxide (NO); Oxyradical; Glucocorticoid receptor; TGF-β1 (transforming growth factor-β1)

1. Introduction

Glucocorticoid shows anti-inflammatory and immunosuppressant effects in various human diseases and experimental animal models. Many effects of glucocorticoids occur after a lag time (1–3 h) during which directly acting anti-inflammatory protein(s) such as macrocortin (called lately as lipocortin) (Blackwell et al., 1980), and/or vasoregulin (Oyanagui and Suzuki, 1985) are synthesized. Cyclosporin A and FK506 (tacrolimus) are immunosuppressants which are now generally used during organ transplantation in combination with a glucocorticoid. These drugs are believed to block interleukin-2 production by T-lymphocytes and thus prevent graft rejection. This blocking action depends on inhibition of the calcium-regulated phosphatase calcineurin after formation of a complex [FK-binding protein-12]-FK506 (Liu, 1993) or a complex [cyclophilin-40]-cyclosporin A (Bram et al., 1993). However, it is reported that the complications associated with cyclosporin A (fibrosis and hypertension) cannot be explained in terms of the interleukin-2 blocking theory (Prashar et al., 1995). In an in vitro study, FK506 enhanced the expression of murine mammary tumor virus-chloramphenicol acetyltransferase in L929 cells when given with 10^{-8} or 10^{-7} M dexamethasone (Ning and Sanchez, 1993). This 50-fold increase in acetyltransferase expression was also observed with 10^{-6} M FK506 or 5×10^{-6} M cyclosporin A in fibroblast cells with 10^{-8} M dexamethasone (Renoir et al., 1995). These recent in vitro data urged me to look for a mechanism of dexamethasone action in vivo which could be modified by immunosuppressants. Dexamethasone (0.01–3 mg/kg, s.c.) alone does not suppress histamine-induced edema in mice at 1 h after its injection but thereafter suppresses edema in a dose-related manner with a maximum at 3 h. Nevertheless, a significant suppression was observed as early as 30 min after dexamethasone injection when the animals were pretreated with FK506 (10 mg/kg, oral) (Oyanagui, 1994). FK506 seemed to enhance the production of anti-in-

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flammatory protein(s) by accelerating the activation of the glucocorticoid receptor, thereby increasing the entry of the active glucocorticoid receptor complex and its binding to the enhancer site on DNA. Other immunosuppressants were also tested in order to know whether the accelerating effect of edema suppression was specific for FK506 or could be generalized to all immunosuppressants. The ischemic paw edema model is a better model of the situation of transplanted organs than the histamine edema model is, so the ischemic edema model was adopted in this report. Transforming growth factor-β1 (TGF-β1) null mutation mice have inflammation of numerous organs (Kulkarni et al., 1993). This cytokine is an immunosuppressant by itself and prolongs the suppression of ischemic paw edema induced by dexamethasone in FK506-treated mice (Oyanagui, 1996). In the study by Oyanagui (1996), dexamethasone suppressed histamine-induced paw edema at 3 h after dexamethasone in normal mice, at 30 min after dexamethasone in FK506-treated mice and at 6 h after dexamethasone in FK506 plus transforming growth factor-β1-treated mice. This suppression was impaired by nitric oxide (NO) synthase inhibitor (N^G-nitro-L-arginine methyl ester: L-NAME), superoxide (O_2^-) scavenger (Cu,Zn-superoxide dismutase: SOD), H_2O_2 scavenger (catalase), hydroxyl radical ($\cdot$OH) scavenger (mannitol) and protein synthesis inhibitor (cycloheximide). Therefore, endogenous NO, O_2^-, H_2O_2 or $\cdot$OH is essential for the suppression of edema by dexamethasone with or without FK506 and transforming growth factor-β1. It was tested in this report using the ischemic paw edema model whether this impairment elicited by these inhibitors and scavengers can be observed in animals treated with other immunosuppressants, namely cyclosporin A, rapamycin and deoxyspergualin.

2. Materials and methods

2.1. Animals and assay

Male ddY mice were obtained from SLC (Shizuoka, Japan) and kept in an air-conditioned room at least for 1 day. They were weighed 1–2 h before the experiment, for which mice weighing 30–36 g were used. The right hind paw was bound with a commercial rubber ring (1×1 mm, $d = 42$ mm) to induce ischemia. The effect of applying the rubber ring for various times (8–14 times) just above the articulation to cause different durations of ischemia (5–120 min) was investigated, as was the effect of different durations of natural blood recirculation (5–180 min) after removal, by cutting, of the rubber ring. After 20 min of recirculation, the average paw swelling was 0.63, 0.81, 0.94 and 1.23 mm after, 5, 20, 60 and 120 min ischemia elicited by applying the ring 12 times. After 20 min ischemia, the average paw swelling was 0.48, 0.81, 1.01, 1.03 and 0.98 mm after 10, 20, 30, 60 and 120 min of recirculation (Oyanagui et al., 1988). In another study, application of the ring 10 times induced similar time-dependent swelling but seemed to be better for evaluating the drug action. Thus ischemic paw edema provoked by 20 min ischemia with 10 applications of the rubber ring followed by 20 min of recirculation was adopted in this experiment for evaluating the drug-induced suppression or enhancement of paw edema. The rubber ring was applied under light ether anesthesia, according to the ethical guide lines of the Japanese Experimental Animal Association. Details of the manipulation using a plastic cylinder device were as in a previous report (Oyanagui and Sato, 1990). The increase in paw thickness (not circumference) was obtained as the difference in thickness measured with the Citizen Thickness gauge (Citizen Watch, Tokyo) before and after the ischemic insult. The average increase in paw thickness in control animals was 0.77 mm to 0.84 mm ($n = 5$, 32 experiments). Assays were performed at 1, 2, 3, 5, 6 and 18 h after dexamethasone. Transforming growth factor-β1 was first injected just before the immunosuppressant, which was generally injected 30 min before dexamethasone (0.1 mg/kg, s.c.). This dose of dexamethasone was the best for testing the effect of other drugs, as determined in a preliminary study of the effects of 0.01–10 mg/kg dexamethasone. All drugs were given as solubilized or well suspended solution (0.2 ml/kg body weight). The increased paw thickness of drug-treated mice ($n = 3$) was compared with the average value of control ($n = 5$) on each experimental day for calculation of suppression. Tests for one dose were repeated 4 times on different days (12 mice in total) and the suppression is presented as mean $\pm$ S.E. (standard error). Statistical significance was determined by using Student's paired *t*-test. The average suppression produced by the vehicles for FK506, rapamycin and deoxysperugalin ($< 7\%$) or for cyclosporin A (olive oil, $< 20\%$) was subtracted before calculating the suppression produced by a drug.

2.2. Drugs and vehicles

Dexamethasone (Decadron®, 4 mg/ml as phosphate ester) was purchased from Banyu Pharmaceutical (Tokyo). Transforming growth factor-β1 (recombinant human, 10^6 u/mg) was obtained from Seikagaku (Tokyo). These drugs were diluted with saline. FK506, cyclosporin A and rapamycin were extracted in this laboratory. FK506 (tacrolimus, from *Streptomyces tukubaenensis*) contained vehicle for oral use (lactose and hydroxypropyl methyl cellulose 2919) of 4 times by weight basis. Cyclosporin A (from *Trichoderma polysporum*) was dosed orally as olive oil solution. One mg of rapamycin (from *Streptomyces hygroscopicus*) was dissolved in 0.8 ml ethanol containing detergent HCO-60 (200 mg) and made to 10 ml with saline. Deoxyspergualin (Spanidin®, Nihon Kayaku, Tokyo) was a lyophilized powder of 100 mg deoxyspergualin · 3HCl and 200 mg lactose. Dose was calculated as

deoxyspergualin · 3HCl. N^G-nitro-L-arginine methyl ester (L-NAME) and Cu,Zn-superoxide dismutase (bovine erythrocyte, 3000 u/mg solid) were provided by Sigma (St. Louis). Catalase (bovine liver, 1500 u/mg solid) was a product of Tokyo Kasei (Tokyo). Desferroxamine mesylate (Desferal®) was obtained from Ciba-Geigy (Takarazuka). Other chemicals were of analytical grade and obtained from Nakalai Tesk Chem. (Tokyo). Indomethacin was dissolved in *N,N*-dimethylformamide and diluted with saline to give a final concentration of this solvent of 10%. Ten percent *N,N*-dimethylformamide alone did not suppress by more than $4 \pm 3\%$ the edema measured in non-treated and dexamethasone-treated mice at 1 and 6 h ($n = 10$, each).

3. Results

3.1. Time-course of edema suppression

Dexamethasone alone (0.01–3 mg/kg, s.c., i.p., i.v., oral) showed no significant suppression of ischemic and histamine paw edema when dexamethasone was given 1 h in advance (data not shown). Dexamethasone is believed not to suppress edema directly, and at least one hour is required to induce anti-inflammatory proteins under normal conditions. The peak of suppression was at 3 h with a potency of $7 \pm 4\%$, $20 \pm 3\%$, $38 \pm 3\%$, $46 \pm 3\%$, $60 \pm 2\%$ for 0.01, 0.1, 0.3, 1 and 3 mg/kg dexamethasone (s.c.), respectively. FK506 (0.1 mg/kg, oral) or cyclosporin A (1 mg/kg, oral) alone produced slight suppression only at 2–6 h, perhaps due to the contribution of endogenous glucocorticoids (Fig. 1B). Transforming growth factor-β1 is reported as an anti-inflammatory and immunosuppressant compound, but showed no effect by itself in these experimental models at doses of 0.1–3 μg/kg (Fig. 1A for 0.3 μg/kg). Dexamethasone (0.1 mg/kg, s.c.) produced a significant suppression at 1 h after its injection in FK506 (0.1 mg/kg, oral)- or cyclosporin A (1 mg/kg, oral)-treated mice. The suppression seen between 2–6 h was increased by FK506 or cyclosporin A treatment (Fig. 1(A)). Transforming growth factor-β1 (0.3 μg/kg, i.p.) with dexamethasone (0.1 mg/kg, s.c.) produced suppression with a peak at 2 h ($47 \pm 5\%$), but FK506 (0.1 and 1 mg/kg) plus transforming growth factor β1 or cyclosporin A (1 and 10 mg/kg) plus transforming growth factor β1 was not suppressive up to 18 h, except for a small effect of FK506 at 6 h (Fig. 3). The most remarkable role of transforming growth factor-β1 was to prolong the suppressive effects of dexamethasone plus FK506 and dexamethasone plus cyclosporin A up to 18 h in addition to causing suppression at 1 h (Fig. 1B). Almost similar data were obtained for histamine-induced paw edema (assayed at 13 min after 3 μg/10 μl histamine in a paw) (data not presented). Hydrocortisone (0.03–10 mg/kg, s.c.) with FK506 (10 mg/kg) suppressed ischemic and

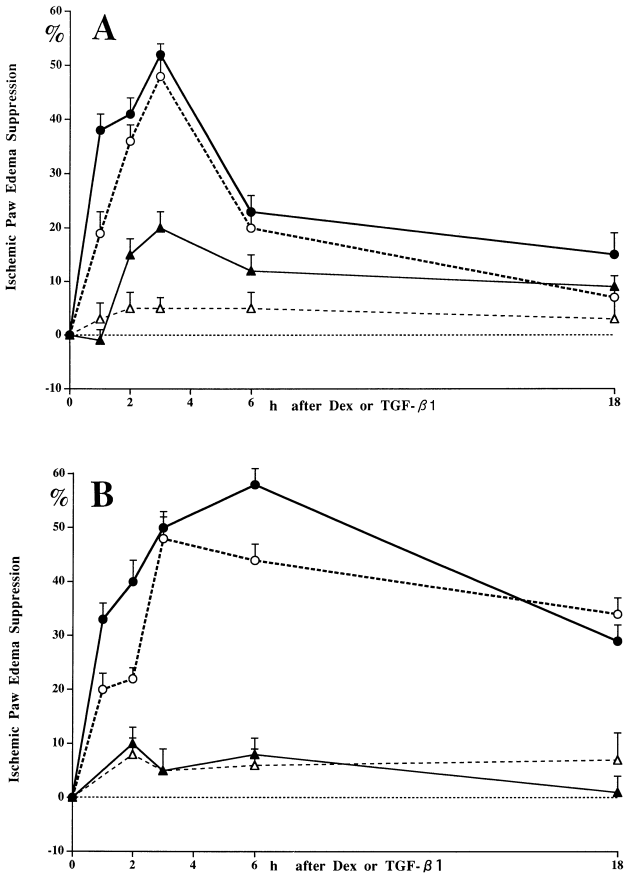

Fig. 1. Suppression of ischemic paw edema by dexamethasone (0.1 mg/kg), FK506 (FK, 0.1 mg/kg, oral), cyclosporin A (1 mg/kg, oral) and transforming growth factor-β1 (0.3 μg/kg, i.p.), alone or in combination. (A) FK506 (●) or cyclosporin A (○)-treated mice showed an accelerated (1–3 h) and increased (1–6 h) suppression by dexamethasone. Dexamethasone alone (▲) did not cause suppression until 1 h and 20% suppression (maximum) at 3 h. Transforming growth factor-β1 alone (△) did not have a significant effect. (B) Transforming growth factor-β1 increased and prolonged the suppression seen in FK506 plus dexamethasone-treated (●) or cyclosporin A plus dexamethasone-treated (○) mice up to 18 h after dexamethasone injection. FK506 alone (▲) and cyclosporin A alone (△) did not have significant effects. Data represent the mean ± S.E. ($n = 12$).

histamine paw edema at 30 min after its injection although an about 15-fold higher dose than that of dexamethasone was required (Oyanagui, 1994).

Dexamethasone (0.1 mg/kg, s.c.) suppressed ischemic edema by $38 \pm 3\%$, $50 \pm 2\%$ and $9 \pm 3\%$ in rapamycin (0.1 mg/kg, oral)-treated mice, and by $4 \pm 3\%$, $44 \pm 3\%$ and $15 \pm 4\%$ in deoxyspergualin (1 mg/kg, i.p.)-treated mice, at 1, 3 and 6 h after dexamethasone, respectively (data not in figure). However, when these mice were treated additionally with transforming growth factor-β1 (0.3 μg/kg, i.p.), the suppression produced by rapamycin at 6 and 18 h after dexamethasone was $50 \pm 5\%$ and $29 \pm 3\%$, and that produced by deoxyspergualin was $55 \pm 2\%$ and $37 \pm 3\%$ (Fig. 2). Rapamycin or deoxyspergualin alone never suppressed edema by more than 10% throughout the observation period.

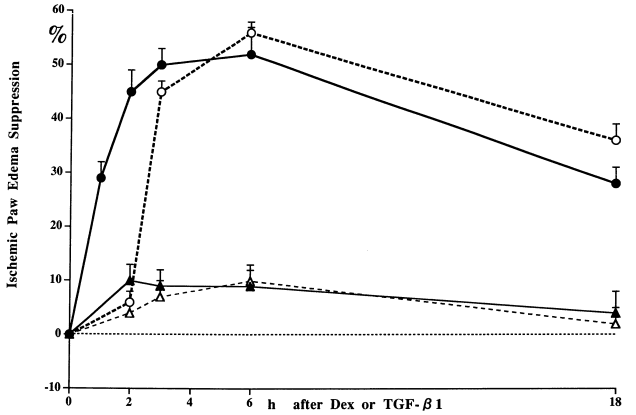

Fig. 2. Suppression of ischemic paw edema by dexamethasone (0.1 mg/kg), rapamycin 0.1 mg/kg, oral) or deoxyspergualin (1 mg/kg, i.p.) with or without transforming growth factor-β1 (0.3 μg/kg, i.p.). Rapamycin-treated (●) mice showed an accelerated (1–3 h) and prolonged (1–18 h) suppression produced by dexamethasone. Deoxyspergualin-treated (○) mice showed only prolonged suppression by dexamethasone (3–18 h). Rapamycin alone (▲) and deoxyspergualin alone (△) did not have significant effects. Data represent the mean ± S.E. ($n = 12$).

Dexamethasone was injected at 5 h instead of at 0 h in order to examine the latent capacity of FK506 or cyclosporin A in combination with transforming growth factor-β1 (0.3 μg/kg, i.p.) to suppress ischemic edema (Fig. 3). There was no or little suppression when the drugs were tested at 5 or 6 h in (0.1 mg/kg FK506 plus transforming growth factor-β1)- or (1 mg/kg cyclosporin A plus transforming growth factor-β1)-treated mice. Nevertheless, when dexamethasone (0.1 mg/kg, s.c.) was injected at 5 h, the suppression rapidly increased after 1 h (at 6 h in Fig. 3) in these animals.

3.2. Dose–response curve of immunosuppressant

Prolongation of the suppressive effect seemed to be clinically most important, so that the suppression of edema elicited by dexamethasone was compared with the effect of immunosuppressant combined with transforming growth factor β1 at 6 h after dexamethasone. As less than 1 μg/kg FK506 caused a significant suppression of ischemic paw edema, the potencies of the immunosuppressants were ordered according to their 30% suppressive

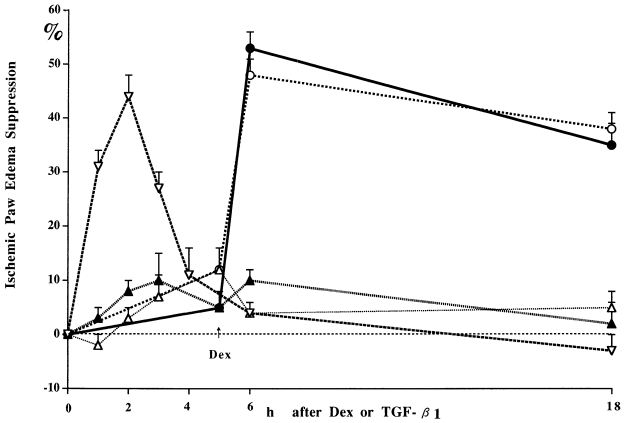

Fig. 3. Suppression of ischemic paw edema by dexamethasone (0.1 mg/kg) injected at 5 h in (0.1 mg/kg FK506 plus 0.3 μg/kg transforming growth factor-β1)-treated (●) or (1 mg/kg cyclosporin A plus 0.3 μg/kg transforming growth factor-β1)-treated (○) mice. There was no significant suppression in (transforming growth factor-β1 plus FK506: ▲)-treated and (transforming growth factor-β1 plus cyclosporin A: △)-treated groups up to 18 h except a slight suppression at 5 h in the latter group. However, a rapid suppression appeared (at 6 h) in these groups when dexamethasone was injected at 5 h (instead of 0 h). This suppression continued at least up to 18 h. An accelerated suppression at only 1–3 h was observed in the (0 h-injected dexamethasone plus transforming growth factor-β1: ▽)-treated group. Data represent the mean ± S.E. ($n = 12$).

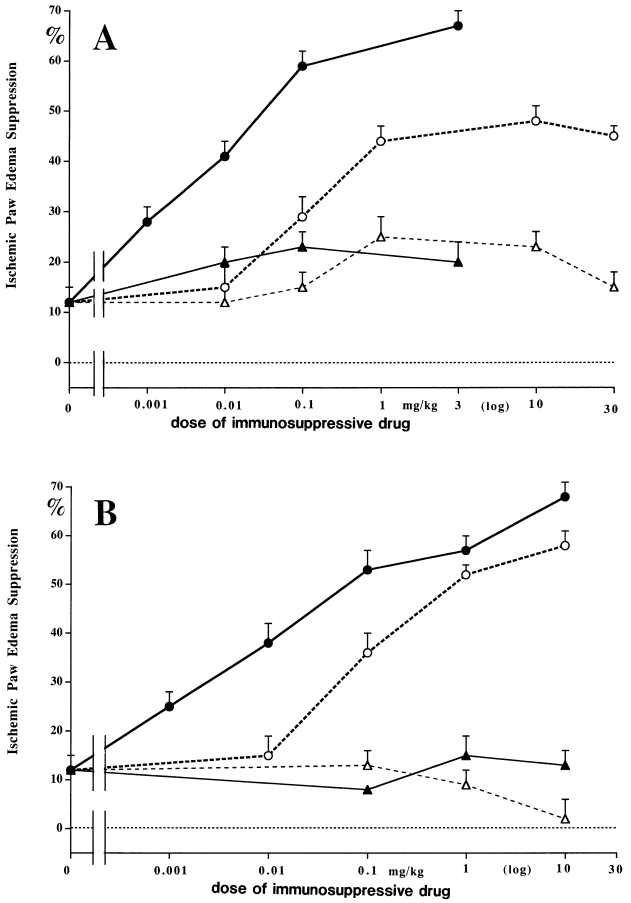

Fig. 4. Suppression of ischemic paw edema by FK506, cyclosporin A, rapamycin or deoxyspergualin with or without transforming growth factor-β1 (0.3 μg/kg) at 6 h after dexamethasone (0.1 mg/kg). (A) FK506 (●) or cyclosporin A (○) significantly suppressed the edema in transforming growth factor-β1 plus dexamethasone-treated mice whereas dexamethasone alone suppressed edema by only 12 ± 3%. FK506 plus dexamethasone (▲) or cyclosporin A plus dexamethasone (△) did not cause statistically significant suppression. (B) rapamycin (●) or deoxyspergualin (○) also suppressed the edema in transforming growth factor-β1 plus dexamethasone-treated mice, whereas rapamycin plus dexamethasone (▲) or deoxyspergualin plus dexamethasone (△) did not increase the suppression of edema. Data represent the mean ± S.E. ($n = 12$).

dose (ED_{30}), namely 1.5 μg/kg FK506 > 3 μg/kg rapamycin > 70 μg/kg deoxyspergualin > 100 μg/kg cyclosporin A (Fig. 4A,B). Higher doses of any immunosuppressant failed to suppress the edema if transforming growth factor-β1 was not co-injected.

3.3. Effects of drugs on suppression by immunosuppressant at 1 h after dexamethasone

The doses of immunosuppressants indicated in Fig. 5 suppressed edema by 40–48% (left in the first rank in Fig. 5) at 1 h after dexamethasone (0.1 mg/kg). The suppression produced by the immunosuppressants alone was less than 10% (right in the first rank in Fig. 5) which may be due to the endogenous glucocorticoids. A typical nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg, i.p.) was a weak suppressor by itself (11 ± 3%), but reversed the suppression by dexamethasone plus an immunosuppressant. A hydrogen peroxide (H_2O_2)-removing enzyme, catalase (30 mg/kg, i.p.), a superoxide radical (O_2^-) scavenger, Cu,Zn-superoxide dismutase (30 mg/kg, i.p.) and hydroxyl radical ($\cdot$OH) scavenger, mannitol (100 mg/kg, i.p.) were more potent to impair the suppression of edema. A protein synthesis inhibitor, cycloheximide (10 mg/kg, i.p.), always blocked the suppression of edema, demonstrating that the main factor in the suppression of ischemic paw edema is dexamethasone, which promotes the synthesis of unknown anti-inflammatory protein(s). Immunosuppressants may simply fortify this effect of dexamethasone. Desferal is a typical iron chelator and might have impaired iron-requiring $\cdot$OH generation from O_2^- and H_2O_2. This suggests that endogenous $\cdot$OH contributes to the suppression of ischemic paw edema. Indomethacin (an inhibitor of prostaglandin synthesis), mepyramine (histamine H_1 receptor antagonist) and cimetidine (histamine H_2 receptor antagonist) did not influence the suppression by dexamethasone with FK506 or cyclosporin A. Prostaglandins and histamine did not participate in the immunosuppressant-induced suppression at 1 h after dexamethasone. The slight suppression produced by mepyramine alone suggests that the histamine H_1 receptor-mediated histamine signal is a little involved in the formation of ischemic paw edema.

3.4. Effects of drugs on suppression by immunosuppressant plus transforming growth factor-β1 at 6 h after dexamethasone

The suppression of paw edema produced by immunosuppressant (indicated dose in Fig. 6) plus transforming growth factor-β1 (0.3 μg/kg, i.p.) at 6 h after dexamethasone (0.1 mg/kg, s.c.) was 47–60%, and 3–17% when

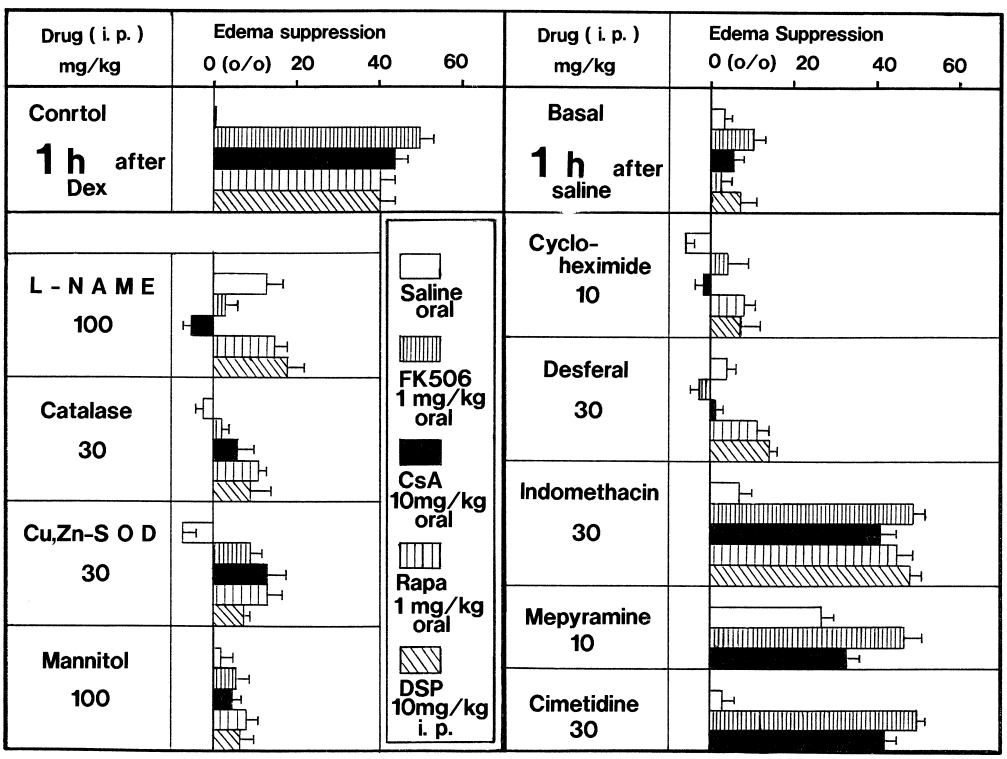

Fig. 5. Effect of inhibitor on ischemic paw edema which was suppressed by immunosuppressant at 1 h after 0.1 mg/kg dexamethasone. Inhibitor was injected 30 min before dexamethasone. The suppression obtained with dexamethasone plus immunosuppressants was 40–50%, whereas that without dexamethasone was 3–10% in control animals. Free radical scavengers, Desferal and cycloheximide reversed the suppression produced by dexamethasone plus immunosuppressants (all with statistically significant difference: $P < 0.01$). Indomethacin, mepyramine and cimetidine had no or little capacity to reverse this suppression. Data represent the mean $\pm$ S.E. ($n = 12$).

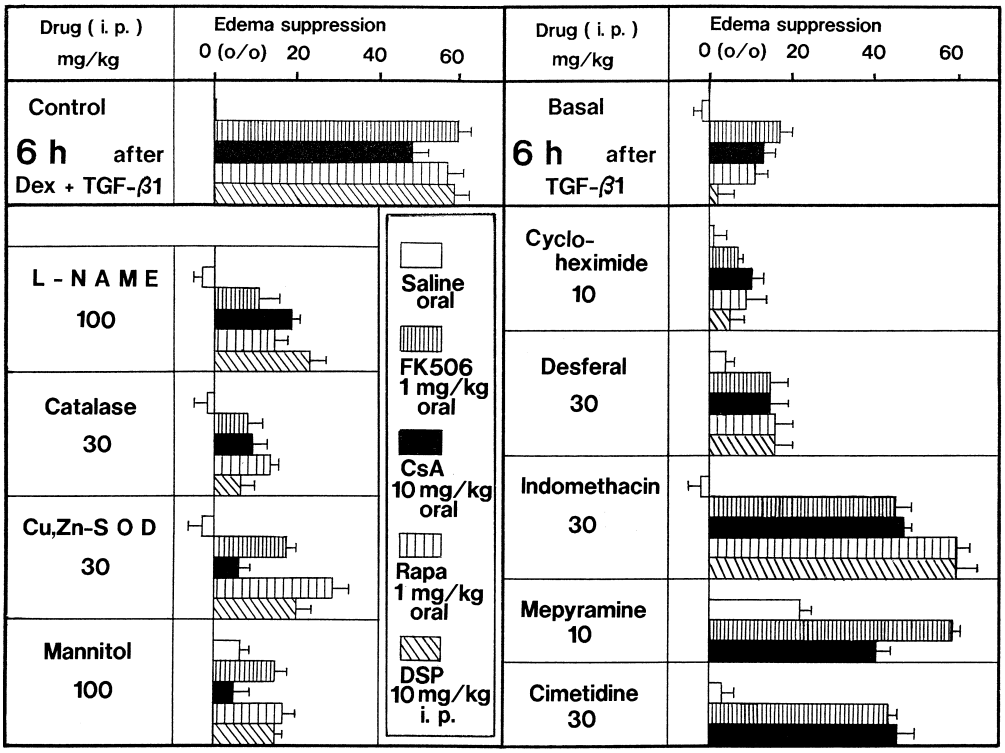

Fig. 6. Effect of inhibitor on ischemic paw edema which was suppressed by immunosuppressant and transforming growth factor-β1 (0.3 μg/kg) at 6 h after 0.1 mg/kg dexamethasone. Inhibitor was injected 30 min before dexamethasone. The suppression produced by dexamethasone, immunosuppressants and transforming growth factor-β1 was 46–59%, whereas that without dexamethasone was 2–16% in control animals. Free radical scavengers, Desferal and cycloheximide reversed the suppression produced by dexamethasone + immunosuppressant + transforming growth factor-β1 (all with statistically significant difference: $P < 0.01$). Indomethacin, mepyramine and cimetidine had no or little capacity to reverse this suppression. Data represent the mean ± S.E. ($n = 12$).

dexamethasone was not injected (in the first rank of Fig. 6). The effect of drug together with transforming growth factor-β1 at 6 h after dexamethasone was slightly weaker than the effect without transforming growth factor β1 at 1 h after dexamethasone. This must be due to the time-dependent decay or excretion of drug. In fact, three separate injections (-30 min, 2 h and 4 h after dexamethasone) of the same total dose did cause a greater impairment of edema suppression at 6 h in the case of Cu,Zn-superoxide dismutase and mannitol (data not shown). NO, H_2O_2, O_2^-, $\cdot$OH, protein synthesis and/or trace iron ion seemed to be required to suppress the edema at 6 h after dexamethasone. Indomethacin and histamine antagonists were without effects on edema suppression at 6 h after dexamethasone in this protocol (Fig. 6).

4. Discussion

The suppression produced by dexamethasone requires protein synthesis in serotonin-induced paw edema of mice (Turufuji et al., 1979), and I found the same results for histamine-, bradykinin- and ischemia-induced paw edema (Oyanagui, 1981). Oxygen radical scavengers, inhibitors of nitric oxide synthesis and non-steroidal anti-inflammatory drugs (NSAIDs) were co-injected with dexamethasone with the expectation that they would modify the effect of dexamethasone. However, Cu,Zn-superoxide dismutase, catalase, mannitol, Desferal, reduced glutathione (GSH), ascorbate, N^G-nitro-L-arginine methyl ester (L-NAME), diclofenac sodium, indomethacin, ibuprofen, mefenamic acid, adenosine, histidine and melatonin, failed to change the suppression produced by dexamethasone of histamine- and ischemic-paw edema of mice. These drugs alone were also inactive in modifying paw swelling (data not shown). An immunosuppressant, FK506 (0.01–10 mg/kg, oral), was the first drug to have an early effect on the suppression produced by dexamethasone (0.03 mg/kg, s.c.) in these edema models. Dexamethasone alone (0.03–10 mg/kg, s.c., oral, i.p.) required at least 2 h for suppression (the time needed for synthesis of anti-inflammatory protein(s)), but when 10 mg/kg FK506 (oral)-treated mice were used, the suppression produced by 0.03 mg/kg dexamethasone (s.c.) appeared as early as 15 min after dexamethasone and was followed by enhanced suppression of histamine and ischemic paw edemata up to 6 h. The suppression of histamine edema by 0.03 mg/kg dexamethasone plus 10 mg/kg FK506 was 38%, 54% and 52% at 30 min, 3 h and 6 h after dexamethasone, respectively. FK506 alone (10 mg/kg) suppressed edema by 13%, 16%

and 9% at these corresponding times, perhaps due to the endogenous glucocorticoids. Hydrocortisone showed the same pattern of suppression in FK506-treated mice, but needed an about 15-fold higher dose than that of dexamethasone (Oyanagui, 1994). A low dose of FK506 (≤ 1 mg/kg) was potent to suppress both histamine and ischemic edema at 30 min, but not at 6 h after dexamethasone injection. However, co-injection of transforming growth factor-β1 (0.1–3 μg/kg, i.p.) suppressed the edema at 6 h after dexamethasone (0.3 mg/kg, s.c.) in FK506 (1 mg/kg, oral or i.p.)-treated mice. Pretreatment with a mixture of transforming growth factor-β1, β2, β3-antibodies blocked completely the suppression-enhancing effects of transforming growth factor-β1 on histamine and ischemic edema in FK506 plus dexamethasone-treated mice (Oyanagui, 1996). Transforming growth factor-β1 was also effective to reducing tumor necrosis factor-α (3 mg/kg, i.p.)-enhanced paw swelling produced by histamine and ischemia in dexamethasone-treated mice. Intraperitoneally injected transforming growth factor-β1 is possible to reach the paw because this cytokine can be transferred during lactation (corresponds oral dosing) to avoid inflammation of various organs in transforming growth factor-β1 null mice (Letterio et al., 1994).

In this report, other immunosuppressants were also demonstrated to enhance the appearance of suppression of ischemic paw edema in dexamethasone-treated mice and to prolong the suppression time in dexamethasone plus transforming growth factor-β1-treated mice.

This study is one of the first to demonstrate in vivo that immunosuppressants enhance the actions of glucocorticoids in addition to the claimed interleukin-2 (IL-2) decreasing action of FK506, cyclosporin A or rapamycin, and the anti-proliferative effect of deoxyspergualin against lymphocytes. The action of steroid is classified in three types (Dean and Sanders, 1996), namely (A) primary response – steroid-bound steroid receptor (SR) complex enters the nucleus and attaches to the enhancer site of DNA and produces a functional protein in several minutes without synthesis of new intermediator protein (for example, c-Fos). (B) Secondary response – steroid-bound glucocorticoid receptor complex produces a protein required for transcription of the secondary response gene which attaches to the enhancer site of DNA and produces a functional protein in several hours or days (for example, ovalbumin, arginase). (C) Delayed primary response – (A + B) type; The attachments of both steroid-bound steroid receptor and primarily synthesized protein to DNA is required for functional protein production in several hours or days (for example, tryptophan oxygenase). In this model of ischemic paw edema, no suppression was observed until 1 h after dexamethasone injection, so that the A type mechanism can be excluded. However, it was difficult to clarify which mechanism (B or C) is involved in these experiments in vivo. The accelerated suppression of edema by immunosuppressants at 1 h after dexamethasone was cycloheximide-sensitive (Fig. 5). This protein synthesis inhibitor might directly block the synthesis of finally acting anti-inflammatory proteins such as vasoregulin (Oyanagui and Suzuki, 1985). Immunosuppressants seem to modify the conformational change or to stabilize the cytosolic dexamethasone-bound glucocorticoid receptor complex (Ning and Sanchez, 1995) and facilitate the dissociation of hsp 90 or the entrance of active dexamethasone-bound glucocorticoid receptor complex into the nucleus. Modified dexamethasone-bound glucocorticoid receptor complex may also attach easily or firmly to the enhancer site of DNA. FK506 and rapamycin bind to hsp 56 of glucocorticoid receptor complex (Yem et al., 1992). Cyclosporin A might bind to cyclophilin 40 (CyP-40) of a recently discovered other type of glucocorticoid receptor complex (Owens-Grillo et al., 1995). Deoxyspergualin is reported to bind both hsp 70 and hsp 90 (Nadeau et al., 1994). The suppression-enhancing effect of deoxyspergualin appeared a little later (3 h) than the effects produced by the other immunosuppressants (1 h), perhaps because of a difference in the hsp in the glucocorticoid receptor complex to which the drug binds. The change in conformation of the glucocorticoid receptor complex must result in a rapid appearance and general enhancement of edema suppression via accelerated transcription of mRNA for unidentified anti-inflammatory protein(s).

FK506 and rapamycin primarily bind to FK506 binding protein-12 (FKBP-12) to reduce the interleukin-2 (IL-2) production, but the natural ligand for FK506-binding protein-12 has not been identified. One candidate as natural ligand is transforming growth factor-β1 receptor I (Wang et al., 1994). Transforming growth factor-β1 binds to transforming growth factor-β1 receptor-I, and it is possible that this complex has an increased ability to react with FK506 binding protein-12. FK506 or rapamycin might have stronger affinity for FK506-binding protein-12 than free transforming growth factor-β1 receptor-I, but there is no data about the reactivity of FK506 or rapamycin when transforming growth factor-β1 receptor-I is bound to transforming growth factor-β1. On the basis of the results of this study, a complex with 3 components (transforming growth factor-β1, transforming growth factor-β1 receptor-I and FK506 binding protein-12) could bind a component (hsp) of the glucocorticoid receptor complex and possibly activate it. It is also possible that FK506 binding protein-12 is one component of the glucocorticoid receptor complex because this receptor complex still has unidentified heat shock protein (hsp) components. Transforming growth factor-β1 is a multifunctional cytokine that has immunosuppressive and anti-inflammatory actions as well as fibrosis-stimulating action depending on the condition. Its null mutation in mice causes an excessive inflammatory response and early death (Kulkarni et al., 1993). The concentration of transforming growth factor-β1 in serum or in paw was not measured in this study, but its active form in the serum of volunteers is reported to be 3.7 ± 3.4 ng/ml

for men and 5.3 ± 4.7 ng/ml for women (Grainger et al., 1995). The amount of transforming growth factor-β1 (0.3 μg/kg) injected in the active form corresponded to 9.9 ng/33 g body weight or 9.9 ng/2.54 ml in blood (= 3.9 ng/ml) when the blood volume was considered as 1/13th of body weight. This dose did not greatly exceed the physiological range.

These data can be understood only by proposing a mechanism as follows: FK506 and rapamycin may amplify the edema suppression produced by dexamethasone by two routes at least (via hsp 56 binding and via FK506 binding protein-12 binding). In the latter case, there must be competition between FK506 or rapamycin and transforming growth factor-β1-bound transforming growth factor-β1 receptor-I. In fact, 10 mg/kg FK-506 produced edema suppression at 6 h after dexamethasone, but 1 or 0.1 mg/kg FK506 failed to suppress edema at 6 h without co-injection of transforming growth factor-β1. The rapid appearance of suppression in FK506 or cyclosporin A plus transforming growth factor-β1-treated mice produced by dexamethasone injected at 5 h instead of at 0 h (Fig. 3) demonstrated that glucocorticoid receptor complex binding to DNA is essential for edema suppression. The animals that received dexamethasone at 0 h with FK506 or cyclosporin A followed by transforming growth factor-β1 injection at 5 h showed no suppression at 6 and 18 h. When dexamethasone (0.1 mg/kg) was injected at 0 h in transforming growth factor-β1-treated mice (no immunosuppressant) there was an increased suppression at 2 h but the animals did not respond to the second injection of the same dose of dexamethasone at 5 h (data not shown). This failure to respond to the second dexamethasone injection may be due to the lack of recycled inactive glucocorticoid receptor complex which can bind dexamethasone in the cytosol. The suppression of edema by dexamethasone in transforming growth factor-treated mice reached a peak at 2 h though the maximum suppression produced by dexamethasone alone was at 3 h. The reason for this difference is not clear, but this cytokine serves as a switch between pro- and anti-inflammatory actions, namely excess suppression by dexamethasone was possibly blocked by the pro-inflammatory action of transforming growth factor. This reversal function elicited by this cytokine could be abolished when an immunosuppressant was concomitantly injected. These results exclude the possibility that transforming growth factor-β1 is a primary intermediate protein in the B or C type steroid mechanism classified by Dean and Sanders (1996) for the induction of functional anti-inflammatory protein(s). Transforming growth factor-β1 (TGF-β1) is an immunosuppressant, and therefore it might have a temporary edema-suppressing action in the absence of other immunosuppressant (FK506 etc.). The rapid appearance of edema suppression produced by dexamethasone in immunosuppressant-treated mice is theoretically interesting and was dose-dependent in the range of 0.01–10 mg/kg FK506, 0.01–3 mg/kg dexamethasone or 0.03–10 mg/kg hydrocortisone in histamine and ischemic paw edema (Oyanagui, 1994). However, its clinical importance may lie in the prolongation of the effect of dexamethasone by a low dose of immunosuppressants plus transforming growth factor-β1. Low doses of dexamethasone and/or an immunosuppressant surely decrease their specific adverse effects. The order of potency to increase the suppression of edema at 6 h after dexamethasone was FK506 > rapamycin > deoxyspergualin > cyclosporin A, although their advantages and disadvantages should be considered when choosing an immunosuppressant. Possible increased adverse effects by the combination of these drugs is an important subject for future study, but the adverse effects characteristic for each drug must be diminished, as less than one-tenth dosing of each drug caused the same suppression when they were given in combination.

The glucocorticoid receptor complex is one of the transcription factors which has cross-talk with other transcription factors such as immunoglobulin κ light-chain binding nuclear factor (NF-κB) or activator protein-1 (AP-1) (Vig et al., 1994). As the role of NK-κB and AP-1 can be influenced by the redox state (Schenk et al., 1994), it is possible that the activity of the glucocorticoid receptor complex can be influenced by the redox state. I examined the effects of anti-oxidant enzymes, NO synthase inhibitor and iron chelator on edema suppression which was enhanced by dexamethasone and immunosuppressant with or without transforming growth factor-β1. There is a theory that the main effect of glucocorticoid depends on the suppression of NF-κB which produces various inflammatory cytokines. Nevertheless, the data for a short time test (20 min + 20 min) or 13 min could be interpreted by excluding the effect of cross-talk between glucocorticoid receptor complex and other transcription factor(s), although a part of the suppression by dexamethasone is due to the enhanced synthesis of IκB (a natural component to be blocking NF-κB action). The reduced suppression of edema at 1 h after dexamethasone produced by inhibitors such as N^G-nitro-L-arginine methyl ester (L-NAME), catalase, Cu,Zn-superoxide dismutase, mannitol and Desferal suggested that endogenous NO, H_2O_2, O_2^-, and/or $\cdot$OH is (are) essential for edema suppression by dexamethasone plus an immunosuppressant. Protein synthesis is needed for dexamethasone action, because cycloheximide abolished completely the suppression of edema. As indomethacin, mepyramine and cimetidine were without effect, prostaglandins and histamine are not involved in suppression by dexamethasone in ischemic paw edema (Fig. 5). Almost the same suppression was obtained with these inhibitors at 6 h after dexamethasone in transforming growth factor-β1-treated mice (Fig. 6). Therefore, both at 1 h and 6 h after dexamethasone, the free radical most likely to participate directly in suppression of edema seems to be $\cdot$OH because it can be generated both via the $O_2^- - H_2O_2 - Fe^{2+}$ system and from NO via formation of

peroxynitrite ($ONOO^-$). It is also possible that some free radicals act directly, but it is not possible to detect directly the free radical species active in the ischemic edema. The exact steps(s) at which $^{\cdot}OH$, oxyradicals or NO works is also difficult to determine. Possible steps are: (1) dissociation of hsp 90 to activate the glucocorticoid receptor complex in the cytosol, (2) penetration of active glucocorticoid receptor complex into the nucleus, (3) attachment of active glucocorticoid receptor to the glucocorticoid response element (GRE) in the DNA chain. The post-transcriptional participation of NO (Xie and Nathan, 1994) cannot be ignored in future studies. The results of this study await confirmation in experiments with adrenalectomized and RU486 (mifepristone, glucocorticoid receptor antagonist)-treated mice.

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