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Mouse Thymocyte Cytolysis of Several Anti-inflammatory Steroid Derivatives

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Abstract□For evaluating the cytolytic effects on the mouse thymocytes, four typical anti-inflammatory steroids (dexamethasone, triamcinolone acetonide, prednisolone, hydrocortisone) were selected in this study. When steroids were treated to the mouse thymocytes *in vitro* cytolysis occurred with dose-dependent fashion and the activities were found to be parallel with the known local anti-inflammatory activities. *In vivo* thymus atrophogenic activities appeared by the treatment of topical and subcutaneous applications of the derivatives were also found to dose-dependent, but not coincided with the thymocyte cytolytic activities *in vitro* and local anti-inflammatory activity in the case of triamcinolone acetonide. Triamicinolone acetonide induced potent thymocyte cytolysis in vitro, but showed less thymus atrophy.

Keywords□Anti-inflammatory steroid, thymocyte cytolysis, thymus atrophy, systemic effect, local anti-inflammtory activity.

Since hydrocortisone was found to be effective against rheumatoid arthritis,[1] the anti-inflammatory glucocorticoid derivatives have been clinically used to treat the inflammatory and immune malfunctional diseases. As a result of the various chemical and microbiological modifications, anti-inflammatory activity was greatly increased and sodium retention was successfully abrogated. However, the unwanted systemic side-effects, mainly glucocorticoidal activisty, may not be avoided because glucocorticoidal and anti-inflammatory activities are believed to be mediated by binding to the same cellular glucocorticoid receptor. To overcome this problem, recent efforts toward discovery of compounds demonstrating separation of local anti-inflammatory activity from potentially harmful systemic side-effects have increased using the characteristics of metabolism, excretion rate, membrane transport of the individual steroid derivative[2-4].

Among the various parameters demonstrating systemic side-effects such as growth retardation, PA axis suppression and liver glycogen deposition, thymus atrophy is very sensitive and easily measured parameter in the experimental glucocorticoid-sensitive species. Thymus atrophy occurs via metabolic inhibition and cytolysis of thymocytes by glucocorticoids. When anti-inflammatory steroids were treated at the high concentrations, thymocytes from glucocorticoid-sensitive strain were known to be gradually cytolyzed *in vitro*.[5-7] It is considered that the influx of extracellular Ca^{++} activates Ca^{++}-dependent DNase and the activated DNase cuts chromosomal DNA and finally leads to cytolytic cell death[5,6]. The cytolytic activities were previously reported to be roughly parallel with anti-inflammatory activities of the steroid derivatives, at least in dexamethasone and hydrocortisone[7]. Therefore, it may be interesting to investigate the thymocyte cytolytic activity of the other derivatives and it seems to be valuable to compare with *in vivo* thymus atrophogenic activity and anti-inflammatory activity. The discrepancies obtained between *in vitro* thymocyte cytolytic activity, *in vivo* thymus atrophogenic activity and anti-inflammatory activity might give a insight for the development of safer local anti-inflammatory steroid derivatives.

In this study, as a part of our continuing research for the development of safer local anti-inflammatory steroids, *in vitro* thymocyte cytolytic activity was evaluated with four steroid derivatives. And the results were compared with *in vivo* thymus atrophogenic activity and the known anti-inflammatory activity.

MATERIALS AND METHODS

Materials

Hydrocortisone and dexamethasone were purchased from Sigma Chem. Co. (St. Louis, MO). Pred-

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nisolone was a product of Upjohn Co. (Kalamazoo, MI). Triamcinolone acetonide (TA) was obtained from Steraloids Inc. (Wilton, NH). RPMI 1640, fetal calf serum (FCS), Pen-Strep. sol'n. (10,000 IU/ml, 10,000 g/ml), Hepes, L-glutamine and 7.5% sod. bicarbonate were purchased from Flow Lab. The other reagents used were reagents grade or cell culture grade.

Mice (C57BL/6, ICR) were maintained with standard mouse pellet lab. chow. (Sam Yang Co.) and tap water *ad libitum* in the animal room (College of Pharmacy, KNU) under conditions of 21-26°C, 40-50% humidity and 12 h/12 h (L/D) cycle.

In vitro cytolysis of mouse thymocytes

After head dislocation of C57BL/6 mice (♂, 21-26 g), the thymus tissues were exercized under aseptic condition. The thymus was rinced in 10 ml of cold incomplete RPMI 1640 and transferred to fresh RPMI media (10 ml). The isolated thymocyte were obtained by teasing with two microscopic slides. The solution was transferred to plastic cell culture tube (Falcon) and left for 5 min. The supernatents were centrifuged at 4°C, 1,000 rpm for 5 min and the thymocyte pellet was obtained. After contaminated RBCs were lysed with the treatment of NH_4Cl sol'n, an aliquote of complete RPMI 1640 (10% FCS, 2 mM glutamine, 1% Pen. - Strep) was added and the cells were resuspended. The number of thymocytes were counted with hemacytometer (Improved Naubauer chamber) and the viability was checked with 0.4% trypan blue sol'n following the procedure of Patterson[8]. After proper dilution, the thymocyte were transferred to 96-well cell culture plate (Flow Lab.), 3×10^5 cells/200 μl/well. The various concentrations of steroid derivatives in ethanol were added to each well (2 μl). The wells considered as control received ethanol (2 μl) only. The plate were transferred to CO_2-incubator (Vision Sci. Co., Korea) and maintained in 5% CO_2/95% air at 37.0±0.2°C.

Thymus atrophy In vivo

In order to evaluate the thymus atrophogenic activity, the various concentrations of steroid derivatives were dissolved in acetone and administered to ICR mice (♂, 23-30 g) in two different routes. For topical administration, the steroid in 50 μl acetone were applied to both ears (25 μl each) of mice. After maintaining in the animal room with standard lab chow and water for 0-9 days, the mice were sacrificed and the thymus tissue were weighed. For subcutaneous injection, the steroid solutions in 100 μl acetone were administered. After 3 days, the mice were sacrificed and the thymus tissues were weighed. For above both experiments, same amount of acetone was applied to the control groups of mice.

RESULTS AND DISCUSSION

The potencies of local anti-inflammatory activities of the selected derivatives in this study were known to be 1 for hydrocortisone, 4 for prednisolone, 25 for dexamethasone and 40-100 for TA depending on the routes of administration[9]. In order to compare these activities with *in vitro* thymocyte cytolytic activity, mouse thymocytes were incubated with various concentrations of the steroid derivatives. The thymocytes isolated from C57BL/6 mice were found to possess over 95% viability using trypan blue dye exclusion test. To determine proper incubation time, the thymocytes were incubated with the fixed concentration of the steroid derivaties (10^{-7} M) for various time intervals. As shown in Fig. 1, the thymocytes were rapidly cytolyzed up to 9 hrs of incubation and after 20-24 hrs, the death rates were not further increased significantly. Therefore, it was determined that 24 hrs of incubation may be adequate to compare the cytolytic activity of the steroid derivatives. When the viability was examined, more than 90% of cells among the remainings thymocytes were always found to exclude trypan blue at any incubation time indicated. And the control wells receiving only ethanol (2 μl) showed 3-9% cytolysis and viability also exceeded 90%.

The viability obtained in this study was not coincided with the other published results[7,10]. But it is thought that the discrepancies in viability were probably due to the different species used and/or the differ-

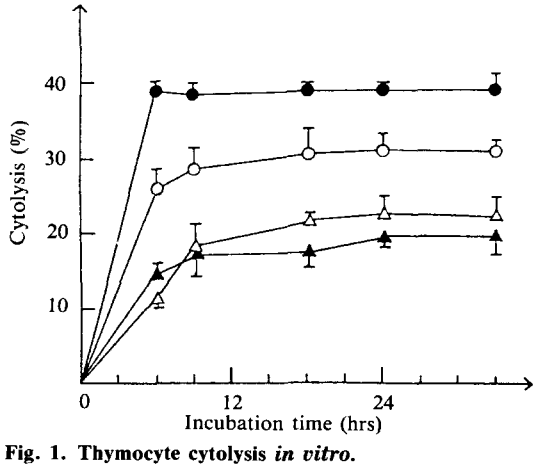

Fig. 1. Thymocyte cytolysis *in vitro*.
The steroid derivatives (10^{-7} M) were used. Dexamethasone (-○-), triamcinolone acetonide (-●-), prednisolone (-△-), hydrocortisone (-▲-)., Data points and bar represent mean±SD of 8 wells.

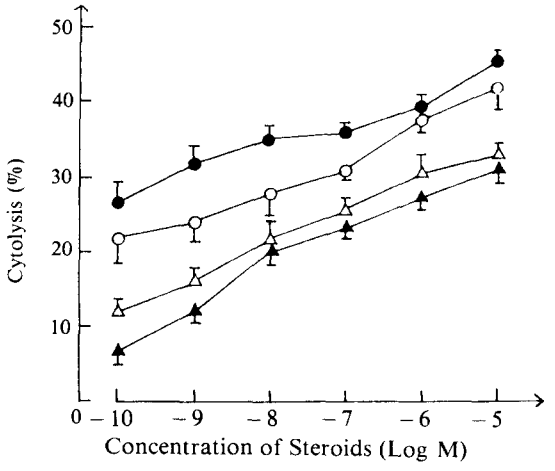

Fig. 2. Thymocyte cytolysis *in vitro* with the derivatives
Dexamethasone (○), triamcinolone acetonide (●), prednisoline (△) and hydrocortisone (▲) were incubated for 24 hrs. Data points and bar represent mean ± SD of 8 wells.

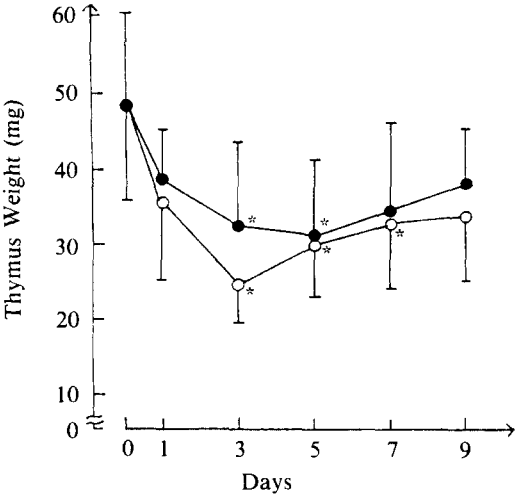

Fig. 3. Thymus atrophy by topical administration of dexamethasone and triamcinolone acetonide (TA)
Dexamethasone (0.05 mg, ○) and TA (0.07 mg, ●) were applied. Data points and bar represent mean ± SD of 8 mice. Statistically significant from thymus wt. (0 days), $p<0.05$ (*).

ent culture conditions such as serm concentration and media composition. Leung and Munck[7] incubated rat (SD) thymocytes in Medium 199 supplemented with 0.12% methyl cellulose. When they treated with 10^{-6} M hydrocortisone for 24 hrs, 61% of thymocytes were appeared to be viable. McConkey *et al*[10] used RPMI 1640 plus 1% bovine serum albumin with 10^{-6} M methyl prednisolone. After 18 hrs of incubation, only 15% of rat (SD) thymocytes were viable. Actually, when we cultured the thymocytes in serumless media, more then 40% of thymocytes were cytolyzed and only 45-55% cells were found to be viable after 24 hrs. Next, 10^{-10} to 10^{-5} M concentrations of the steroid derivatives were incubated for 24 hrs. Fig. 2. represented the dose-dependent cytolysis of mice (C57BL/6) thymocytes. As expected, the order of potencies of cytolysis was well correlated with the local anti-inflammatory activities of the steroid oderivatives (TA > dexamethasone > prednisolone > hydrocortisone). These results suggested that *in vitro* thymocyte cytolysis test might be a useful mean to evaluate the intrinsic local anti-inflammatory activity of the derivatives. In order to match these *in vitro* results with *in vivo* thymus atrophogenic activity, the topical and subcutaneous administrations of the derivatives were carried out. When dexamethasone and TA were topically applied to ICR mice, thymus weights were rapidly decreased within 3-5 days and recovered slowly there after (Fig. 3).

From this experiment, it was demonstrated that 3-5 days are adequate to measure the thymus atrophogenic activity for the derivatives. As the various amounts of the derivatives were applied topically, dose-depentent decreases in the thymus weights were found as shown in Table I. The order of thymus atrophogenic activities of the derivatives were same as the order of *in vitro* thymocyte cytolysis except TA. In this experiment, dexamethasone showed higher activity than TA in contrast to the local anti-inflammatory activity and *in vitro* thymocyte cytolytic activity. From this study, it was demonstrated that *in vitro* thymocyte cytolytic activity was parallel with local anti-inflammatory activity, but it was not matched with the degree of systemic effects (*in vivo* thymus atrophogenicity). To exclude the possibility showing differences occurred by the different skin pernetration rate of the derivatives, subcutaneous injection was also tried. Table II showed the thymus atrophogenic activities of the derivatives after SC injection. The results were very similar as in topical application, which clearly indicated that TA showed less thymus atrophogenic activity *in vivo* (systemic effects).

From these results, it is thought that the low thymus atrophogenic activity mediated by TA is not due to the low intrinsic activity to thymocytes or difficulties of skin penetration rate. The reasons of low systemic activity of TA are presumably due to the different pharmacokinetic properties such as fast biotrasformation, protein binding and percutaneous absorption rate[4,11]. Therefore, it could be possible to deve-

Table I. Thymus atrophy by topical administration of the steroid derivatives

Group[1]	Dose (mg/mouse)					
	0.01	0.02	0.05	0.07	0.10	0.50
Dexamethasone	79.2 ± 11.9*[2,3]	60.5 ± 20.8*	61.2 ± 17.2**	43.8 ± 15.0**	57.7 ± 10.1**	47.4 ± 8.8**
TA	98.2 ± 14.3	83.0 ± 28.9	78.1 ± 13.6*	69.7 ± 27.0*	59.0 ± 7.4**	46.9 ± 11.4**
Prednisolone	107.3 ± 8.9	101.6 ± 17.2	91.1 ± 10.8	–	76.7 ± 27.5	53.3 ± 13.5**
Hydrocortisone	–[4]	–	103.8 ± 12.2	–	96.1 ± 12.2	57.7 ± 16.6**

[1]. All compounds dissolved in 50 μl acetone were subcutaneously applied to ICR mice (n = 8/gruop) and sacrificed after 3 days.
[2]. Values represents percent of mean ± SD compared with control group, 100.0 ± 15.8% (35.3 ± 5.6 mg thymus wt.).
[3]. Statistically significant from control by student t-test, *: $p<0.05$, **: $p< 0.001$.
[4]. Not tested.

Table II. Thymus atrophy by subcutaneous injection of the steroid derivatives

Group[1]	Dose (mg/mouse)			
	0.01	0.02	0.05	0.07
Dexamethasone	61.3 ± 16.7**[2,3]	50.2 ± 12.5	44.1 ± 10.6**	–
TA	88.3 ± 16.1	79.8 ± 14.6*	51.4 ± 11.9**	–
Prednisolone	–[4]	108.6 ± 13.3	73.5 ± 21.4*	65.0 ± 10.7**
Hydrocortisone	–	101.9 ± 16.5	97.0 ± 12.7	84.8 ± 11.3*

[1]. All compounds dissolved in 100 l acetone were subcutaneously applied to ICR mice (n = 8/gruop) and sacrificed after 3 days.
[2]. Values represent percent of mean ± SD compared with control group, 100.0 ± 11.1% (34.1 ± 3.8 mg, thymus wt.).
[3]. Statistically significant from control by student t-test, *: $p<0.05$, **: $p<0.001$.
[4]. Not tested.

lope safer local anti-inflammatory steroid by designing the compounds having advantageous pharamacokinetic properties, although it might not be possible to seperate anti-inflammatory activity from unwanted glucocorticoidal activity at the receptor level. Based on this kind of concept (fast metabolic inactivation), "antedrug", several compounds are now under investigation for development of safer local anti-inflammatory steroids[3,12].

In conclusion, TA showed high thymocyte cytolytic activity (*in vitro*) with low thymus atrophogenic activity (*in vivo*) amoung the four studied derivatives. Thymocyte cytolysis test combined *in vivo* thymus atrophogenic test employing mice may be simple and rapid methods for evaluating local anti-inflammatory activity and systemic effects of known and/or new steroid derivatives.

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