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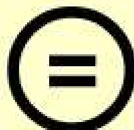

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Effect of Cyclosporin A on Human Melanocytes

아주대학교 대학원

의 학 과

이 지 연

Effect of Cyclosporin A on Human Melanocytes

지도교수 강 원 형

이 논문을 의학 석사학위 논문으로 제출함.

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심사위원장 강 원 형 인

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심사위원 임 선 빈 인

아 주 대 학 교 대 학 원

2000 년 12 월 23 일

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항상 많은 격려와 도움을 주신 부모님께도 마음속 깊이 감사의 마음을 전합니다.

2000 년 12 월
저자 씀

- ABSTRACT -

Effect of Cyclosporin A on Human Melanocytes

Cyclosporin A (CsA) is a potent immunosuppressive agent and has been used to be effective in the treatment of psoriasis, atopic dermatitis.

Until now the effects of Cyclosporin A (CsA) on skin pigmentation have never been investigated. We investigated the effect of CsA on the proliferation and pigmentation of cultured human melanocytes. In this study, we first investigated the direct effects of CsA on proliferation and tyrosinase activity of cultured human melanocytes. In addition, we investigated the tyrosinase synthesis by western blot analysis and northern blot analysis.

CsA decreased the cell number of cultured melanocytes at concentrations of 10^{-7} M to 10^{-5} M in a dose-dependent manner. Tyrosinase activity was not decreased by 10^{-7} M and 10^{-6} M CsA, but was decreased in the presence of 10^{-5} M CsA. The production of tyrosinase and tyrosinase related protein-1 (TRP-1) at both the mRNA and protein level was decreased at concentrations of 10^{-7}M to 10^{-5}M in a dose-dependent manner. These results suggest that CsA may inhibit the proliferation of melanocytes and their synthesis of tyrosinase and TRP-1 at therapeutic

concentrations, but not inhibit the pre-existing tyrosinase activity at concentrations of 10^{-7} M and 10^{-6} M.

Key Words: Melanocyte, Cyclosporin A, Tyrosinase, Pigmentation

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Effect of Cyclosporin A on Human Melanocytes

Ji Yeoun Lee

Department of Medical Sciences

The Graduate School, Ajou University

(Directed by Professor Won Hyoung Kang)

I. INTRODUCTION

Cyclosporin A (CsA), a cyclic undecapeptide, is a powerful immunosuppressant widely used in the prevention of graft rejection. Cyclosporin A (CsA) is also effective in the treatment of some patients with various autoimmune diseases, and in some patients with psoriasis, atopic dermatitis, and other immune skin disorders.[1,2]

The major intracellular binding protein for Cyclosporin A (CsA) is

cyclophilin, which is ubiquitously distributed in eukaryocytes and have peptidyl-prolyl cis-trans isomerase activity. The target of these CsA-cyclophilin complexes has been identified as calcineurin, a calcium calmodulin-dependent protein phosphatase, although the binding and inhibition of calcineurin do not require calmodulin. It is well established that CsA exert its effects by interacting with cytosolic receptor protein, cyclophilin which inhibits calcineurin, resulting in the inhibition of nuclear translocation of transcription factors such as nuclear factor of activated T cell (NF-AT).[3,4,5]

Skin color mainly depends on the deposition of melanin pigment in the epidermis. Melanin production is principally responsible for skin color and plays an important role in prevention of sun-induced skin injury.[6] Melanin pigment in the epidermis is present in keratinocytes and melanocytes. Since melanin in the keratinocytes is derived from melanocytes, evaluation of factors affecting pigmentation has been performed by examining the melanin content or tyrosinase activity in melanocytes. Melanin is produced in melanocytes, neural crest-derived cells residing at the basal layer of epidermis; tyrosine and L-dihydroxyphenylalanine (L-dopa) serve as precursors for this complex biopolymer.[6,7] These precursor amino acids are oxidized by a rate-limiting enzyme tyrosinase[8,9] and subsequent reactions involving tyrosinase and tyrosinase-related proteins result in deposition of melanin pigment in melanosomes. Research on the regulation of melanogenesis has focused on factors affecting tyrosinase, which catalyzes the rate-limiting step of melanogenic

pathway, the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). However, recent studies have indicated that there are additional factors affecting other steps in this pathways, including the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by tyrosinase related protein-2 (TRP-2) as DOPAchrome tautomerase (DT), the oxidation of DHICA by tyrosinase related protein-1 (TRP-1) as DHICA oxidase; the polymerization of DHICA by pmel17/silver locus protein as DHICA polymerase, stablin, etc.[10,11,12]

The relationship of the pigmentation and CsA remains controversial. Brady et al. has described hyperpigmentation due to CsA therapy.[13] After renal transplantation, their patient developed hyperpigmentation of the skin during 3 months' therapy of CsA which became increasingly marked and resolved after CsA therapy was discontinued. Smith et al. observed that there was a significant increase in total number of melanocytic nevi in the renal transplantant group compared with control group.[14] There was a significant negative association between the use of CsA and number of nevi. However Szepietowski et al reported that there were no differences in melanocytic nevi counts in patients treated with or without CsA.[15]

Until now the effects of CsA on skin pigmentation have never been investigated. In this study, we first investigated the direct effects of CsA on proliferation and tyrosinase activity of cultured human melanocytes.[16,17] In addition, we investigated the tyrosinase and tyrosinase related protein-1 (TRP-1) synthesis by western blot analysis and northern blot analysis.

Considering the importance of CsA for its therapeutic use against pigmentary disorders such as melasma, freckles, senile lentigines, vitiligo etc., we have further investigated its effects on tyrosinase and tyrosinase related proteins in the melanin synthetic pathway to clarify the mechanism of action in human melanocytes in culture. Such studies may enlighten the possibility of enhancing CsA's effect on hyperpigmentary or hypopigmentary disorders.

II. MATERIALS AND METHODS

A. Chemicals

Cyclosporin A (CsA), isobutylmethylxanthine (IBMX), cholera toxin, phorbol 12-myristate 13-acetate (PMA), basic fibroblast growth factor (b-FGF) were purchased from Sigma (St. Louis, MO). Bovine pituitary extract (BPE), F-12 nutrient mixture, heat-inactivated fetal bovine serum and penicillin/streptomycin were purchased from Gibco-BRL (Gaithersburg, MD).

B. Human Melanocyte Culture

Normal human melanocytes were isolated from neonatal foreskins as described previously,[18] and were maintained in F-12 nutrient mixture supplemented with 24 ug/ml IBMX, 100 ng/ml cholera toxin, 50 ng/ml PMA, 4% heat-inactivated fetal calf serum, 0.6 ug/ml b-FGF and 1% penicillin/streptomycin with 5% CO_2 at 37°C. The medium was changed every other days. For experiment, melanocytes at third to forth passages were used.

C. Effect of Cyclosporin A on Melanocyte Proliferation

For count of cell numbers, melanocytes were plated at a density of $0.5X10^4$ cells/cm^2 in 60 mm culture dish (Corning, Coning, NY). After 1 day of culture, the medium was replenished and fresh medium containing 0, 10^{-7}, 10^{-6}, 10^{-5} M CsA was added every other day for a total 6 days. At the end of each experiment, melanocytes from each individual dish were harvested and counted using a Coulter counter (Model ZM, Coulter Electronics, Hialcah, FL, England).

D. Effect of Cyclosporin A on Tyrosine Hydroxylase Activity

Human melanocytes were plated at a density of $0.5X10^4$ cells/cm^2 in 60 mm culture dish and cultured as described above for the cell count. Tyrosinase activity was measured according to Pomerantz et al.[19] During the final 24 hour of the experiment, [3,5-^{3}H] L-tyrosine (specific activity, 52 mCi/mmol; Dupont NEN, Boston, MA) at a concentration of 0.7 uCi/ml (total 2.8 uCi/4ml/dish) was added to the culture medium, and the conditioned medium from each dish was saved to be assayed for the activity of tyrosinase, the rate-limiting enzyme in the melanogenic pathway. To measure ^3H_2O release, 1ml of the medium was mixed with activated

charcoal in 0.1M citric acid and the mixture was centrifuged for 10min at 2000g at 4°C. The supernatants were applied to 0.2 ml columns of Dowex-50 equilibrated in 0.1 M citric acid, washed with 1 ml of 0.1 M citric acid, and the effluents were counted by scintillation spectrometry.

E. Western blot analysis of tyrosinase and TRP-1

For western blot analysis of tyrosinase and TRP-1, human melanocytes were plated at a density of $0.5X10^4$ cells/cm^2 in 100 mm culture dish. Western blot was performed for tyrosinase as described by Jimenez et al.[20] After 1 day of culture, the medium was replenished and fresh medium containing 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M CsA was added every other day for a total 6 days. At the end of each experiment, melanocytes from each individual dish were harvested and cell lysates were prepared using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and 50 nM Tris, pH 8.0) containing the phosphatase inhibitor Na_3VO_4 (10 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (200 mM), aprotinin (10 ug/ml), and leupeptin (10 ug/ml). Equal amounts of protein (8 ug) were loaded on each lane and separated by electrophoresis on a 7.5% polyacrylamide gel. After transblotting onto nitrocellulose membrane, the membranes were reacted with PEP7h (1:1500), a rabbit polyclonal antibody raised against the COOH terminus of the human

tyrosinase (a gift from Pichard King and William Oetting, University of Minnesota, Minneapolis, MN.) or with TA99 (1:1500), a mouse monoclonal antibody raised against the human TRP-1 (kindly provided by Setaluri Vijayasarsdhi, Wake Forest University, Winstonsalem, NC). The membranes were then reacted with horseradish peroxidase conjugated anti-rabbit IgG (Amersham, Buckinghamshire, England; 1:3000 dilution after incubation with PEP7h) or anti-immunoreactive bands were detected by chemiluminescence, using the Renaissance kit (Dupont NEN, Boston, MA)

F. Northern blot analysis of tyrosinase and TRP-1

For northern blot analysis of tyrosinase and TRP-1, human melanocytes were plated at a density of $0.5X10^4$ cells/cm^2 in 100 mm culture dish and cultured as described above for the cell count. Three culture dishes of 100 mm were used for a single RNA preparation. RNA extractions were performed with Trireagent (Sigma. St. Louis, MO). For Northern blot analysis, 5 ug of total RNA were separated on formaldehyde gels and capillary-transferred to nylon membranes (Nytran super charge, S&S, Dassel, Germany). Filters were prehybridized (2 hr) and hybridized (12-16 hr) at 42°C in 50% formamide, 3X SSC, 0.1 M Tris, 5X Denhardt's and 10% Dextrane sulfate. The pMEL34 containing an 1.8kb cDNA fragment of human

tyrosinase and TRP-1 were purchased from ATCC (Rockvill, Maryland) and cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was generatrd using the polymerase chain reaction.[21] Plasmid were purified by columns (QIAGEN, Hilden, Germany), specific cDNA insertions were cut out by restriction enzyme separated in agarose gels, and recovered by an elution kit (QIAGEN, Hilden, Germany). Probes were labeled with [α-^{32}P] dCTP by the random priming procedure (rediprime, Amersham, Buckinghamshire, England). Non-incorporated nucleotides were separated by columns (Micro Bio-spin 30: chromatography columns, Bio-Rad, Hercules, CA). The mRNA lengths were determined according to an RNA length markers (0.24-9.5 kb RNA ladder, Gibco-BRL, Gaithersburg, MD). The membranes were washed first in 2X SSC and 0.5% SDS briefly at room temperature and then for 2 hr at 60°C, with frequent changes of wash buffer.[22] Membranes were then exposed to film (Kodak X-Omat) between intensifying screens and stores at -70°C for 1-4 days before developing.

G. Statistical analysis

Statistical significance was tested with oneway ANOVA test, and $p < 0.05$ was taken to be significant.

III. RESULTS

A. Effect of Cyclosporin A on Melanocyte Proliferation

To investigate the effects of the CsA on human melanocytes, we first studied effects of CsA on melanocyte proliferation.

The effects of CsA on melanocyte proliferation during 6 days of culture are shown in Fig. 1. The cell numbers in the presence of 10^{-7}, 10^{-6}, and 10^{-5} M CsA decreased to 91.9±4.6, 83.9±4.3 and 39.7±4.3% (mean±SEM, n=5, p <0.05) of the control cells (100%), respectively. Melanocyte proliferation was dose-dependent decrease at CsA concentrations from 10^{-7} M to 10^{-5} M. At a CsA concentration of 10^{-5} M, a decrease cell numbers was not observed compared with the numbers on Day 0. Melanocytes incubated with 10^{-5} M CsA did not detached from the plastic surfaces of the culture dish and did not show morphological changes such as cytoplasmic vacuoles, shrinkage of nucleus.

B. Effect of Cyclosporin A on Tyrosine Hydroxylase Activity

The effects of CsA on tyrosinase activity during 6 days of culture are shown

in Fig. 2. Tyrosinase activity of melanocytes in the presence of 10^{-7} M and 10^{-6} M CsA was increased to 116.3±22.2 and 113.7±7.3% (mean±SEM, n=5, p <0.05) of the control cells (100%) but were not statistically different values from control. However tyrosinase activity in the presence of 10^{-5} M CsA was decreased to 35.5±13.5% (mean±SEM, n=5, p <0.05) of the control cells (100%).

C.Western blot & Northern blot analysis of tyrosinase and TRP-1

Western blot analysis using anti-tyrosinase and TRP-1 antibodies revealed that there was a gradual decrease in tyrosinase and TRP-1 protein at concentrations of 10^{-9}-10^{-5} M of CsA (Fig. 3). To determine if tyrosinase and TRP-1 mRNA levels parallel those of tyrosinase and TRP-1 protein, northern blot analysis was performed. Northern blot analysis of tyrosinase and TRP-1 demonstrated a gradual decrease in tyrosinase and TRP-1 mRNA 10^{-7}-10^{-5} M of CsA (Fig. 4), which was the same result of western blot analysis.

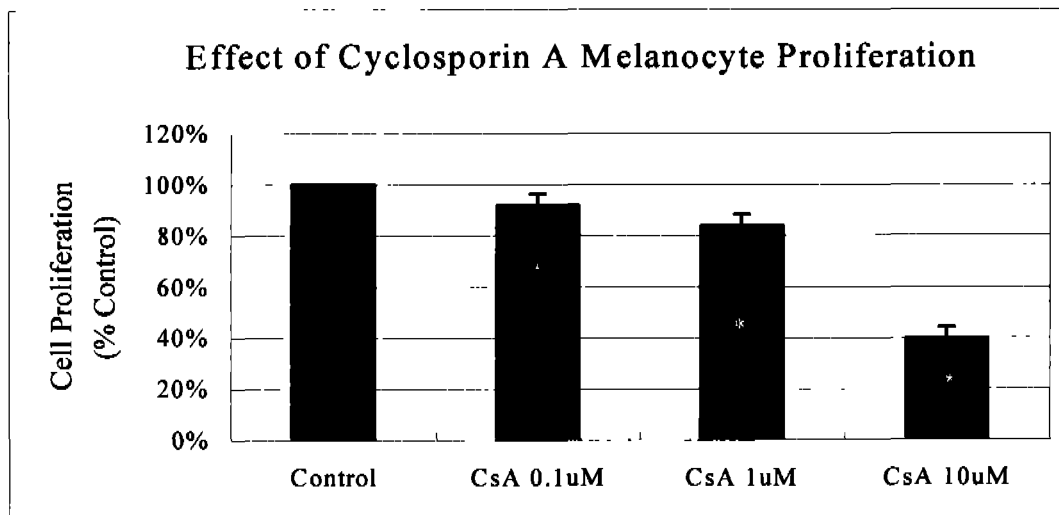

Fig. 1. Effect of CsA on Melanocyte proliferation

Number of cells counted on the 6th day of culture. A significant decrease of cell number was observed as the increased dose of CsA. Five independent experiments were performed and the results are presented as mean ± SEM. The cell numbers in the presence of 10^{-7}, 10^{-6}, and 10^{-5} M CsA decreased to 91.9± 4.6, 83.9± 4.3 and 39.7± 4.3% of the control cells (100%), respectively. Melanocyte proliferation was dose-dependent decrease at CsA concentrations from 10^{-7} M to 10^{-5} M.

*Difference from control: $p<0.05$

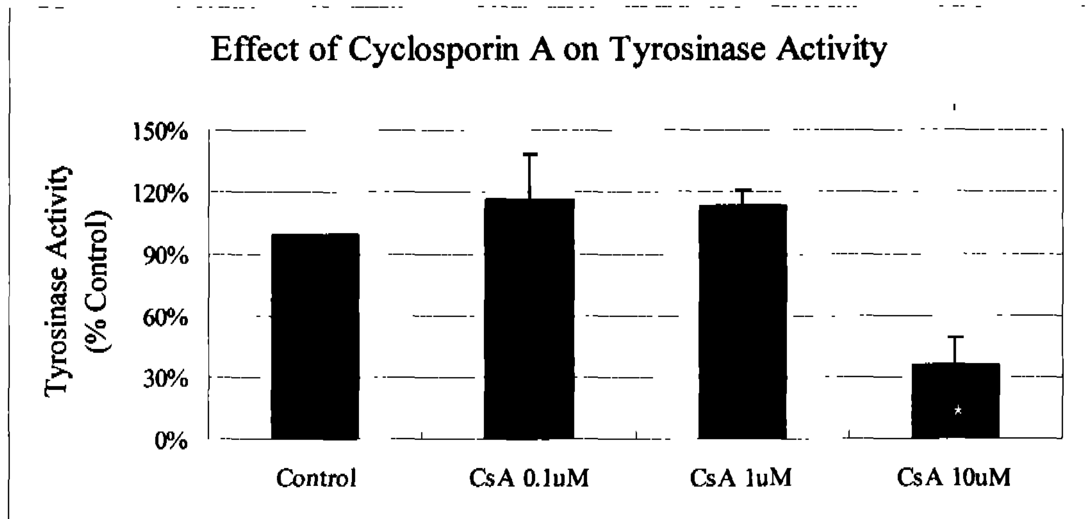

Fig. 2. Effect of CsA on Tyrosinase activity

Tyrosine hydroxylase activity of melanocytes cultured with CsA. Five independent experiments were performed and the results are presented as mean ± SEM.. Tyrosinase activity of melanocytes in the presence of 10^{-7} M and 10^{-6} M CsA was increased to 116.3± 22.2 and 113.7± 7.3% of the control cells (100%) but were not statistically different values from control. However tyrosinase activity in the presence of 10^{-5} M CsA was decreased to 35.5± 13.5% of the control cells (100%).

*Difference from control: $p<0.05$

A.Tyroinase

B.TRP-1

1 2 3 4 5 6

Fig. 3. Western blot analysis of Tyrosinase and Tyrosinase Related Protein (TRP)-1

Regulation of tyrosinase and TRP-1 mRNA levels by CsA. Human melanocytes were cultured and treated with CsA as described. After 6 days cells were collected and protein was isolated and analyzed for tyrosinase and TRP-1 protein content by Western blot analysis. Western blot analysis using anti-tyrosinase and TRP-1 antibodies revealed that there was a gradual decrease in tyrosinase and TRP-1 protein at concentrations of 10^{-9}-10^{-5} M of CsA

A: Tyrosinase B: TRP-1

Lane 1. CsA 0 M treatment (control)

Lane 2. CsA 10^{-9} M treatment

Lane 3. CsA 10^{-8} M treatment

Lane 4. CsA 10^{-7} M treatment

Lane 5. CsA 10^{-6} M treatment

Lane 6. CsA 10^{-5} M treatment

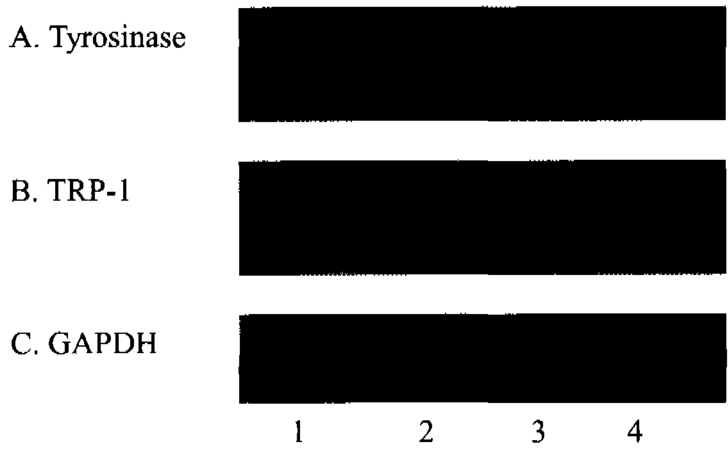

Fig. 4. Northern blot analysis of Tyrosinase and Tyrosinase Related Protein (TRP)-1

Regulation of tyrosinase and TRP-1 mRNA levels by CsA. Human melanocytes were cultured and treated with CsA as described. After 6 days cells were collected and total RNA was isolated and analyzed for tyrosinase and TRP-1 mRNA content by Northern blot analysis. Hybridization of the blot with cDNA for GAPDH, generated using the polymerase chain reaction was used to confirm even loading of lanes. Northern blot analysis of tyrosinase and TRP-1 demonstrated a gradual decrease in tyrosinase and TRP-1 mRNA 10^{-7}-10^{-5} M of CsA

A: Tyrosinase B: TRP-1 C: GAPDH

Lane 1. CsA 0 M treatment (control)

Lane 2. CsA 10^{-7} M treatment

Lane 3. CsA 10^{-6} M treatment

Lane 4. CsA 10^{-5} M treatment

IV. DISCUSSION

There has been no report about the effect of CsA on the proliferation and melanogenensis of human melanocytes.

CsA inhibited melanocyte proliferation

The present study showed that CsA could inhibit the proliferation of cultured melanocytes in a dose-dependent manner at concentrations of 10^{-7} M (0.12 ug/ml) to 10^{-5} M (12 ug/ml). Decrease cell numbers was not observed compared with the numbers on Day 0. Morphological changes, such as cytoplasmic vacuoles, shrinkage of nucleus were not observed in all concentrations. The results suggest that CsA could not be cytotoxic at concentrations of 10^{-7} M to 10^{-5} M.

As to the suppressive effect on cell proliferation, since CsA was found to be effective for treating psoriasis vulgaris, which is characterized by epidermal hyperproliferation with neutrophilic and lymphocytic infiltration, many researchers have explored its effects on epidermal cells. Furue et al[23] reported that CsA inhibited the DNA synthesis and proliferation by normal and transformed keratinocyte cell lines at concentrations of 0.3-10 ug/ml. Sharpe et al[24] reported that b-FGF driven proliferation of cultured human keratinocytes and human umbilical vein endothelial

cells were inhibited by CsA in a dose-dependent manner at concentrations of 0.156 - 5 ug/ml. CsA may interrupt a locally acting paracrine and autocrine growth factor loop mediated by b-FGF. CsA could do this by inhibiting the mitogenic effects of b-FGF on keratinocytes and endothelial cells. Dykes et al[25] observed that CsA inhibited the proliferation of cultured human keratinocytes at concentrations of 1-10 ug/ml (0.83-8.3 uM). Karashima et al[26] reported that CsA inhibited keratinocyte proliferation by blocking cells in the G0/G1 phase of cell cycle. On the other hand, Kato et al[27] found that up to 6 ug/ml of CsA did not inhibit the growth of epidermal cells in a primary organ culture system. Ono et al[28] reported that CsA inhibited the DNA synthesis and cell growth of cultured fibroblasts at concentration of 10^{-8} M to 10^{-6} M in a dose-dependent manner. The production of both collagen and non-collagnenous protein at mRNA and protein was not affected by 10^{-8} M to 10^{-6} M CsA, but was decreased in the presence of 10^{-5} M CsA. Since the inhibitory effect of CsA on the proliferation of certain malignant cell lines of hematopoietic and nonhematopoietic origin has already been reported, it appears that CsA can inhibit the proliferation of various types of cells, although their sensitivity to this compound may vary considerably.

CsA inhibited tyrosinase activity and tyrosinase synthesis

The production of tyrosinase and TRP-1 at mRNA and protein was decreased in a dose-dependent manner at concentrations of 10^{-7} M (0.12 ug/ml) to 10^{-5} M (12 ug/ml). But the rate-limiting enzyme in the melanogenic pathway, tyrosine hydroxylase activity was not affected by 10^{-7} M to 10^{-6} M CsA, but was decreased in the presence of 10^{-5} M CsA. In contrast to murine melanoma cells, no correlation between tyrosinase activity and mRNA in human melanocytes and melanoma cells has been reported by several laboratories. Therefore, it appears from our results that CsA does not inhibit the pre-existing tyrosinase activity except 10^{-5} M CsA but CsA inhibit the tyrosinase and TRP-1 at transcriptional and translational level in human melanocytes in 10^{-7} M - 10^{-5} M CsA.

In this study we found that CsA was effective in inhibiting proliferation of melanocytes and production of tyrosinase and TRP-1. But the pre-existing tyrosinase activity was not inhibited by CsA.

V. CONCLUSION

In this study, we first investigated the direct effects of CsA on proliferation and tyrosinase activity of cultured human melanocytes. In addition, we investigated the tyrosinase synthesis by western blot analysis and northern blot analysis.

The results are as follows.

1. Cyclosporin A inhibited proliferation of cultured human melanocytes at concentrations of 10^{-7} M to 10^{-5} M

2. Cyclosporin A did not inhibit tyrosinase activity of cultured human melanocytes at concentrations of 10^{-7} M and 10^{-6} M, but inhibited tyrosinase activity of cultured human melanocytes at concentrations of 10^{-5} M.

3. Cyclosporin A inhibited the protein synthesis of tyrosinase and TRP-1 proteins at concentrations of 10^{-9} M to 10^{-5} M.

4. Cyclosporin A inhibited the mRNA expression of tyrosinase and TRP-1 proteins at concentrations of 10^{-7} M to 10^{-5} M.

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-국문요약-

싸이클로스포린 A 가 인체 멜라닌세포에 미치는 영향

지도교수 강 원 형

아주대학교 대학원

의학과

이 지 연

싸이클로스포린 A 는 장기이식 후 널리 쓰이고 있는 면역억제제로서 피부과 영역에서는 건선이나 아토피피부염등의 치료에 쓰이는 약물이다. 그러나 현재까지 싸이클로스포린 A 와 피부색소침착의 연관성에 관한 연구는 이루어지지 않았다.

본 연구는 최초의 싸이클로스포린 A 와 피부색소침착의 연관성에 관한 연구로 세포 배양시스템을 이용하여 싸이클로스포린 A 가 인체 멜라닌세포의 증식과 색소형성에 미치는 직접적인 영향을 관찰하였다. 세포 수

의 측정으로 멜라닌세포의 증식에 미치는 영향을 관찰하였고, 티로신 하이드록실라제 활성을 측정하였으며 웨스턴 블로팅과 노던 블로팅을 이용하여 mRNA 와 단백질 레벨에서 티로시나아제 합성에 미치는 영향을 알아보았다.

본 연구 결과 싸이클로스포린 A 는 10^{-7} M 에서 10^{-5} M 농도에서 용량 의존적으로 세포 수를 감소시켰다. 그러나 10^{-7} M 과 10^{-6} M 의 싸이클로스포린 A 는 티로시나아제 활성을 감소시키지 못하였고 10^{-5}M 의 농도에서만 티로시나아제 활성이 감소하였다. 웨스턴 블로팅과 노던 블로팅에서는 싸이클로스포린 A 10^{-7}M 에서 10^{-5}M 로 싸이클로스포린 A 의 농도가 감소하면서 티로시나아제와 티로시나아제 관련 단백질-1 (TRP-1)의 단백질과 mRNA 발현의 감소를 관찰할 수 있었다. 따라서 싸이클로스포린 A 가 다른 질환의 치료에 쓰이는 농도에서 멜라닌세포의 증식과 티로시나아제 합성을 억제 시킬 수 있는 것으로 관찰되었다

핵심되는 말: 멜라닌세포, 싸이클로스포린 A, 티로시나아제, 색소침착