항암제 (paclitaxel) 와 수지상세포 치료법의 병합을 통한 쥐의 섬유육종에 대한 항종양면역치료 방법의 개발

Combined Treatment of Intratumoral Injection of Dendritic Cells and Systemic Chemotherapy (paclitaxel) for Murine Fibrosarcoma

2005년 2월

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이 논문을 박사학위 논문으로 제출함

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이 논문을 박 정 훈의 박사학위논문으로 인정함.

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국문요약

수지상세포는 강력한 항원전달능력이 있고 T세포 면역에 중요한 역할을 담당하고 있어 항암면역치료에 효과적인 세포로 알려져 있다. 최근에 항암 치료와 수지상 세포 종양내 주사치료를 병합하는 새로운 항종양 면역치료 방법이 소개되었으며 효과적인 결과를 보였다. 본 연구에서는 실험동물인 C57BL/6 쥐에 동질 유전자형의 MCA102 섬유육종세포를 주입하여 종양의 생성을 확인한 후, 항암제인 paclitaxel을 투여하고 이어서 수지상세포를 병변내 주사하는 병합치료법을 시행하고 그 효과에 대해서 연구하였다.

실험결과 paclitaxel이나 수지상 세포를 단독으로 치료한 실험군에서는 부분적인 치료효과를 보인 것에 반해 paclitaxel과 수지상세포를 병합치료한 실험군에서는 종양의 우수한 치료 효과를 보였다. 또한 종양이 소멸된 쥐에서 동일한 섬유육종 세포를 다시 주입하더라도 종양의 생성을 완전히 억제해 장기간 지속되는 면역을 획득했음을 알 수 있었다.

결론으로 paclitaxel을 이용한 화학 요법과 수지상 세포치료
법의 병합치료법은 섬유육종의 치료에 효과적인 새로운 항종양면역치료법임을 확인하였으며, 향후 타종양에 대한 치료에도 적용할 수 있음이라 생각된다.

주요어: 수지상세포, Paclitaxel, 병합치료, 항종양면역치료
Abstract

A novel combined treatment of conventional chemotherapy together with intratumoral injection of syngeneic dendritic cells (DCs) has emerged as a potent strategy for cancer treatment. In this study, we evaluated a syngergistic effect of intraperitoneal (i.p.) injection of chemotherapeutic drug, paclitaxel, plus intratumoral (i.t.) injection of syngeneic bone marrow-derived DCs for the treatment of preexisting fibrosarcoma. Subcutaneous tumors were established using MCA102 fibrosarcoma cells in syngeneic C57BL6 mice. The results demonstrate that combined treatment of paclitaxel chemotherapy and injection of DCs led to complete tumor regression, in contrast to partial eradication of the tumors with chemotherapy or DCs alone. Furthermore, the tumor-free mice were able to resist a repeat challenge with the same tumor. These finding suggest that combination therapy of systemic chemotherapy and intratumoral administration of DCs is also potent strategy for the treatment of fibrosarcoma.
Key words: murine fibrosarcoma; dendritic cell; Paclitaxel; immunotherapy; combination therapy
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Fig. 1. Phenotyping of cultured dendritic cells. Primary DCs were obtained from syngeneic mouse bone marrow precursors by culturing in the presence of GM-CSF and IL-4 and then their phenotypes were verified by the flow cytometry. The results showed that DCs isolated were CD11c (B), CD44 (C), and I-A^d (D) positive. On the other hand, CD40 (E) and CD80 (F) expression was very low.

Fig. 2. In vitro cytotoxic effect of paclitaxel on MCA102 murine fibrosarcoma cells and DCs. The cytotoxicity effect of etoposide, ifosfamide, doxorubicine, and paclitaxel on MCA102 tumor cells (A) and that of paclitaxel on DCs (B) was measured by the MTT assay in vitro. Paclitaxel was found to have the most profound cytotoxic effect on MCA102 tumor cells.

Fig. 3. Significant regression of the tumor growth in the combined treatment group in contrast to the other control groups. Using a well
characterized tumor model (MCA102 sarcoma in C57BL/6 mouse), a synergistic effect of a systemic paclitaxel administration by i.p. together with an i.t. injection of DCs was evaluated, compared to chemotherapy or a DC treatment alone. The data represent the means ± SD of three independent experiments.

Fig. 4. Representative photographs of animals showing the tumor masses 15 and 42 days after the tumor cell injection. The mouse treated with the combined therapy showed the most significant suppression of tumor growth. (A, B, C, and D) mice treated with medium, paclitaxel, DC, or paclitaxel plus DC respectively on day 15. (E, F, G, and H) mice treated with medium, paclitaxel, DC, or paclitaxel plus DC respectively on day 42.

Fig. 5. Determination of apoptosis of MCA102 tumor cells in the tumor tissues by the TUNEL assay. Apoptotic cells stained brown were easily observed in the tumor tissues treated with a paclitaxel treatment alone (B), but not in tissues treated with a DC treatment
alone (C) or a medium injection (A). Arrows indicate the apoptotic cells.

Fig. 6. Immunohistochemical staining of the tumor tissue after the combined treatment. When the border areas between the tumor tissue and normal tissue were observed, more immune cells, such as macrophages and neutrophiles, were detected in the mouse specimen from the combined treatment (B) than that from the medium control specimen (A).

Fig. 7. Persistent antitumor memory after the combined treatment. A second challenge of the same MCA102 tumor cells to the mice, in which tumors had completely regressed in the previous experiments, was carried out. A very small size tumor appeared around day 9 after the second injection, but it disappeared quickly thereafter. Age-matched normal mice were also injected with the same type of tumor for controls. The data represent the means ± SD of duplicate experiments.
I. Introduction

Dendritic cells (DCs) are the most effective antigen-presenting cells for T cells, and they have the potential to induce tumor-specific immune responses leading to tumor rejection. DCs capture tumor cells, then generate tumor-specific cytotoxic T lymphocytes (CTLs) from naive T cells to fight against tumor cells (Banchereau et al., 1998; Steinman et al., 1991; Celluzzi et al., 1996). Although tumors have immunogenic epitopes on their surface that can be recognized by the host immune system, failure to mount an appropriate immune response to these antigens results in uncontrolled tumor growth in cancer patients. Immune tolerance to an established tumor is due to the low immunogenicity of the tumor and/or defects in the host immune system (Carbone et al., 1998; Grabbe et al., 1995; Gabrilovich et al., 1996).

Many published trials to overcome tumor tolerance entailed the development of a tumor vaccination that involves injecting DCs, generated and loaded with tumor antigens ex vivo, into cancer
patients (Nestle et al., 1998; Boczkowski et al., 1996; Condon et al., 1996; Gong et al., 2000). When using these approaches, the ex vivo manipulation of the DCs to acquire and present tumor-specific antigens is required. To avoid the requirement of an ex vivo antigen loading process, some groups suggest the administration of an DC injection after a systemic chemotherapy, on the basis that DCs are capable of ingesting apoptotic tumor cells and acquiring tumor-associated antigens, to induce class I-restricted CTLs in vitro (Tong et al., 2001; Inoue et al., 2003; Shin et al., 2003). These trials showed that the antitumor effect was significantly more effective when both chemotherapy and the injection of DC were given.

Paclitaxel is a clinically effective anticancer drug against a variety of cancers. Paclitaxel binds to tubulin, retards microtubule depolymerization, impairs mitosis, blocks cell cycle progression, and facilitates apoptosis (Schiff et al., 1979). Although various chemotherapeutic drugs are used for the combination therapy of DCs and low-dose chemotherapy, paclitaxel had not been challenged for the current trial. Recently, Joo reported that paclitaxel does not
affect the viability of DC (Joo, 2003). In our study, we observed the antitumor effect of a therapeutic trial which is associated with a DC therapy after a low-dose paclitaxel treatment. The results indicate that the i.t. administration of DCs after low-dose chemotherapy via an i.p. injection results in potent and specific antitumor immunity without an ex vivo antigen loading process.
II. Materials and Methods

1. Animals

Female C57BL/6 mice were purchased from Dae Han Bio Link (Korea). The mice were allowed to adjust to their environment for 1 week and they were used at 6-8 weeks of age for DC isolation and at 8-10 weeks of age for tumor inoculation.
2. Tumor Cell Culture

MCA102 is a murine fibrosarcoma cell line of C57BL/6 mouse origin. This cell line was maintained in a complete medium that contained RPMI1640 (Gibco-BRL, Rochville, MD, USA) medium plus 10% heat-inactivated fetal bovine serum (FBS, Gibco-BRL), and 100 U/ml penicillin (GIBCO-BRL) in a 5% CO$_2$ incubator at 37°C to a subconfluent state in 75 cm$^2$ plates. After reaching subconfluence, cells were rinsed twice with phosphate-buffered saline (PBS), or RPMI1640. For detachment, cells grown to confluence were treated with 0.05% trypsin (Gibco-BRL) and 0.53 mM EDTA. For the in vivo inoculation, tumor cells were washed three times and resuspended in PBS.
3. Murine DC Culture

Primary DCs were obtained from mouse bone marrow precursors. Murine bone marrow cells were harvested from femurs and tibias and then plated in complete RPMI1640 containing recombinant murine GM-CSF (10 ng/ml) and recombinant murine IL-4 (10 ng/ml). On day 2, non-adherent granulocytes were gently removed, and fresh medium with GM-CSF and IL-4 was added. On day 4, loosely adherent cells were dislodged and replated. On day 6 of the culture, immature DCs and non-adherent proliferating aggregates were collected and the maturation status and percentage of DCs were verified by flow cytometry with five surface markers (CD11c, CD44, CD40, CD80, and class II antigen I-A^d^), showing the purity of DCs to be $\geq 65\%$. 


4. MTT Assay

The number of viable cells at the end of the culture was quantified by an MTT assay. The tumor cells and DCs in their own complete media were incubated in 6-well plates containing serial dilutions of the chemotherapeutic drugs in a total volume of 3 ml/well and incubated for 48 hr. The concentrations of the employed dilution of etoposide, doxorubicin and ifosfamide were 0.005, 0.01, 0.05, 0.1, 0.5 and 1µg/ml. The concentrations of the employed paclitaxel were 0.005, 0.01, 0.05 0.1, 1, 5, 10 and 50 µg/ml. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma, St Louis, MO, USA), was dissolved in PBS at 5 mg/ml. Following an exposure to the chemotherapeutic drugs, a 1/10 diluted MTT solution (0.5 mg/ml) was added to the assay wells. The plates were incubated at 37°C for 4 hr and the dark blue crystals of MTT-formazan were observed at the bottom of the wells. The formazan precipitate was dissolved with dimethylsulphoxide (DMSO) and mixed in a plate shaker for 20 min. The amount of converted MTT was quantified in an ELISA reader using 570 nm test and 650 nm reference
wavelengths. The results are expressed as the mean ± SD of absorbance.
5. Combined Treatment of Intratumoral DC Injection and Chemotherapy

For the fibrosarcoma MCA102 tumor model, naive C57BL/6 mice were inoculated subcutaneously (s.c.) with $1.5 \times 10^5$ MCA102 cells in the upper right flank. On day 9, when the tumor size reached about 3-5 mm in diameter, paclitaxel (5 mg/kg) was administered i.p. to mice and then the same treatment was given on the 11th and 13th day. DCs were injected i.t. four times on days 10, 12, 14 and 18 ($2 \times 10^6$ cells/mouse for each injection in 50 µl of PBS). This injection protocol is a modification of that described by Shin et al (2003). Four groups (untreated control, DC alone, paclitaxel alone and paclitaxel plus DC) were included and each group had four mice for each treatment. This animal experiment was performed three times. For all animal experiments, the tumor size was measured biweekly using a caliper and expressed as the product of the maximal perpendicular diameter and height (mm$^3$).
6. In vivo analysis of cell death by TUNEL stain

The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. After being washed with PBS, tissue slides were fixed in 10% paraformaldehyde. The TUNEL assay was conducted according to the manufacturer's instructions (TACS In Situ Apoptosis Detection kit, Trevision, CA, USA).
7. Secondary Tumor Challenge in Tumor-free Mice

The persistence of tumor-specific immunity in the mice treated by combination therapy was determined at day 74, 146 after first tumor innoculation. Two mice showing a complete regression of MCA102 fibrosarcoma tumors were given a second s.c. tumor challenge. MCA102 fibrosarcoma tumor cells \( (2 \times 10^5 \text{ cell each in 50} \mu\text{l PBS}) \) were implanted into the left upper flank that was the opposite side to the first injection site. The same number of tumor cells was also injected into age-matched control mice. These mice were followed for survival and measurement of tumor size.
III. Results

Isolation of bone marrow-derived cells and phenotyping

Primary DCs were obtained from syngeneic mouse bone marrow precursors out of femurs and tibias. On day 6 of the culture in the presence of GM-CSF and IL-4, immature DCs were collected and their phenotypes were verified by flow cytometry (Fig. 1). As shown in the Fig. 1, almost no expression of maturity surface markers CD40 and CD80 was detected, indicating that the DCs were in an immature state. Flow cytometry data with three other surface markers, I-A^d, CD11c, and CD44, showed the purity of DCs to be > 65%.

In vitro cytotoxic effect of paclitaxel on murine fibrosarcoma cells and DCs

In order to find out an effective chemotherapeutic agent on MCA102 tumor cells, the cytotoxicity of etoposide, ifosfamide, doxorubicine, and paclitaxel was tested by the MTT assay in vitro.
Among these, paclitaxel showed the most significant cytotoxic effect on MCA102 tumor cells (Fig. 2A). In addition, the cytotoxic effect of paclitaxel on DCs was also tested and was shown to have a lower cytotoxic effect than that on MCA102 (Fig. 2B). These results suggested that paclitaxel would be a good chemotherapeutic agent for this study.

**Significant regression of tumor growth in the combined treatment group in contrast to the other control groups**

To determine whether a systemic paclitaxel administration by intraperitoneal(i.p.) together with an intratumoral(i.t.) injection of DCs has a synergistic effect compared to chemotherapy or a DC treatment alone, a well characterized tumor model (MCA102 sarcoma in C57BL/6 mouse) was evaluated. Paclitaxel chemotherapy was initiated via i.p. when the tumor size was about 4-5 mm² and the i.t. administration of DCs was started 1 day after the first dose of chemotherapy. A detailed injection schedule is described in the Materials and Methods.
As shown in Fig. 3, the tumors grew rapidly in the untreated control mice. The mice treated with paclitaxel or DC alone showed a modest delay of tumor growth. In contrast, the mice treated with the combined systemic paclitaxel administration plus an i.t. injection of DCs showed a significant suppression of tumor growth. These results strongly suggest that combined systemic paclitaxel chemotherapy plus an i.t. injection of DCs has a greater suppression activity on MCA102 sarcoma growth than a paclitaxel or DC alone treatment in vivo. The actual tumor sizes on day 15 and 42 post tumor cell injection are shown in Fig. 4.

Apoptosis of MCA102 tumor cells in tumor tissue after the combined treatment in vivo

To evaluate the apoptosis of MCA102 tumor cells in tumor tissue after each treatment, the TUNEL assay was performed with paraformaldehyde fixed tissue specimens. Apoptotic cells stained brown were easily observed in the tumor tissues treated with the combined treatment and paclitaxel alone, but not as much as in the
tissues treated with DC alone (Fig. 5). These results indicate that paclitaxel injected via i.p. caused apoptosis of MCA102 tumor cells in vivo.

**Immunohistochemical staining of tumor tissue after the combined treatment**

In order to observe the immigration of immune cells to the tumor mass in vivo, an immunohistochemical stain was performed using anti-CD11c. When the border areas between the tumor tissue and normal tissue were observed, more immune cells, such as macrophages and neutrophiles, were detected in each mouse specimen from the combined treatment compared to the control (Fig. 6).

**Persistent antitumor memory after the combined treatment**

To further evaluate the memory of antitumor immunity in vivo after the combined treatment of paclitaxel plus DC, a second challenge of the same MCA102 tumor cells to 2 mice, in which the tumor had
completely regressed, was carried out. As showed in Fig. 7, a very small tumor appeared around day 9 after the injection, but it disappeared quickly in 2 experimental mice. In contrast, as in the previous control experiments, tumors were formed in the 2 control mouse with no treatment.
Fig. 1. Phenotyping of cultured dendritic cells. Primary DCs were obtained from syngeneic mouse bone marrow precursors by culturing in the presence of GM-CSF and IL-4 and then their phenotypes were verified by the flow cytometry. The results showed that DCs isolated were CD11c (B), CD44 (C), and I-A^d (D) positive. On the other hand, CD40 (E) and CD80 (F) expression was very low.
Fig. 2. In vitro cytotoxic effect of paclitaxel on MCA102 murine fibrosarcoma cells and DCs. The cytotoxicity effect of etoposide, ifosfamide, doxorubicine, and paclitaxel on MCA102 tumor cells (A) and that of paclitaxel on DCs (B) was measured by the MTT assay in vitro. Paclitaxel was found to have the most profound cytotoxic effect on MCA102 tumor cells.
Fig. 3. Significant regression of the tumor growth in the combined treatment group in contrast to the other control groups. Using a well characterized tumor model (MCA102 sarcoma in C57BL/6 mouse), a synergistic effect of a systemic paclitaxel administration by i.p. together with an i.t. injection of DCs was evaluated, compared to chemotherapy or a DC treatment alone. The data represent the means ± SD of three independent experiments.
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Fig. 6. Immunohistochemical staining of the tumor tissue after the combined treatment. When the border areas between the tumor tissue and normal tissue were observed, more immune cells, such as macrophages and neutrophiles, were detected in the mouse specimen from the combined treatment (B) than that from the medium control specimen (A).
Fig. 7. Persistent antitumor memory after the combined treatment. A second challenge of the same MCA102 tumor cells to the mice, in which tumors had completely regressed in the previous experiments, was carried out. A very small size tumor appeared around day 9 after the second injection, but it disappeared quickly thereafter. Age-matched normal mice were also injected with the same type of tumor for controls. The data represent the means ± SD of duplicate experiments.
IV. Discussion

The present study demonstrates that a combination therapy of conventional paclitaxel chemotherapy and an i.t. injection of DC is effective for fibrosarcoma, using a C57BL/6 mouse model of flank fibrosarcoma that was syngeneic to the animal. The data showed that the combined treatment resulted in the complete suppression of tumor growth, whereas a single treatment, such as chemotherapy or the DC injection alone, generated only a partial regression of the tumors. In addition, the combined therapy produced tumor-specific cytotoxic T cells that were able to protect animals from a subsequent tumor challenge with the same type of tumor, suggesting that the animals had acquired long-term antitumor immunity. These results suggest that the combination therapy of paclitaxel chemotherapy together with an i.t. injection of syngeneic DC can be a practical method for the treatment of fibrosarcoma.

DC therapy has been considered to be one of the emerging strategies for the treatment of patients with advanced cancer,
especially for patients resisting conventional therapies, such as surgery, irradiation and chemotherapy. Several different strategies have been studied to use DCs in immunotherapy against tumors in the context of DC’s role in presenting MHC-restricted tumor antigens. These strategies include pulsing DCs with defined peptides or a tumor cell lysate, modifying DCs genetically to express tumor antigens, and fusing DCs with tumor cells (Nestle et al., 1998; Boczkowski et al., 1996; Condon et al., 1996; Gong et al., 2000). These manipulated DCs were then reinfused back to the recipient host. These methods can induce CTLs directed specifically against the tumor but they require an ex vivo step of exposing the DCs to the tumor. The strategy used in the present study is similar to the previous strategies but it eliminates the ex vivo step of loading the DCs with a tumor antigen. Instead, our DCs injected into a tumor mass engulfed the apoptotic bodies generated by the paclitaxel treatment directly in vivo. Using this method, we can eliminate any surgical procedures to get tumor tissues, reduce the dose of the chemotherapeutic agent as low as to induce the apoptosis of tumor
cells, and enhance direct contact of DCs to apoptotic tumor cells without migration from remote area. Our observations also suggest that it is not necessary to identify specific tumor antigens for immunotherapy using DCs and low-dose chemotherapy may be a sufficient method for providing antigens to DCs in the milieu of the tumor.

Tong et al and Shin et al have previously reported a similar antitumor strategy as shown in the present study. Tong et al evaluated a synergistic effect of systemic cyclophosphamide chemotherapy combined with an i.t. injection of DCs for the treatment of colon adenocarcinoma and melanoma, and Shin et al tested a synergistic effect of an i.t. injection of combined vincristine and DCs for the treatment of fibrosarcoma. The difference between our method and that of Shin’s group is the following. First, we used a different chemotherapeutic agent, paclitaxel, and this drug was injected i.p. instead of i.t. Second, the injection schedules of the antitumor drug and DCs are different. Basically, we injected DCs 1 day after the paclitaxel injection, whereas Shin’s group injected DCs 8 hours after
a vincristine injection. We hypothesized that there would be more apoptotic cellular bodies available to DCs 1 day after chemotherapy, and a systemic injection of chemotherapeutic drug would generate a higher regression of the tumor. Also, this procedure would lower the treatment burden to the host animals.

DCs are known to be the most potent antigen-presenting cells (APCs) of the immune system. They are capable of activating T cells in response to both new and recall antigens (Banchereau et al., 1998; Steinman et al., 1991; Celluzzi et al., 1996). The maturation state of DC is one of the factors that affect the capacity of DC to induce antigen-specific CTLs. We used immature DCs produced by culturing DC precursors from bone marrow in the presence of GM-CSF and IL-4 for 6 days, because immature DCs have a phagocytic activity and they can perform effective capture of exogenous antigens. However, there is still a need to evaluate how much DCs matured in vitro induce the antitumor effect when combined with low-dose chemotherapy as well as the effects of various routes of a DC injection. Beside these, there are other issues that still remain to be
resolved. (1) Which subset of DCs can stimulate antitumor immunity most effectively? (2) What is the potential for a combination therapy of a DC-based treatment with other therapies (such as radiotherapy or cytokine therapy)? (3) Can a steroid hormone (such as dexamethasone) used in conventional cancer treatment inhibit the anticancer immunity induced by a DC-based treatment?

In summary, a combination therapy of systemic paclitaxel chemotherapy together with an i.t. injection of DCs is a promising method for the eradication of fibrosarcoma in vivo. The data presented in this and other studies strongly suggest that the presented strategy may overcome the problem of drug resistance in conventional chemotherapy and it can be applicable to a wide variety of tumors in clinical settings.
V. References


VI. 감사의 글

부족한 저에게 힘과 지혜를 주셔서 여기에 있게 해주신 하나님께 감사를 드립니다.

먼저 힘든 가운데에서도 기회를 주시고 석사 과정부터 언제나 한결 같은 모습으로 지도와 사랑을 주신 최광성 교수님께 십심한 사의를 표현합니다. 교수님의 첫 제자로서 교수님의 명성에 걸맞는 의사가 되도록 분발하겠습니다. 앞으로도 많은 지도 편달 부탁 드립니다.

논문을 지도해 주시고 학문적 토대를 만들어 주신 송순욱 교수님과 실험을 도와준 연구원들에게도 감사드리며, 바쁘신 중에도 귀한 조언과 더불어 부족한 논문을 성실히 교정하고 지도해 주신 이민걸 교수님, 이광훈 교수님, 이문희 교수님께도 고마움을 전합니다.

이제 과정을 마무리하면서 과거의 올타리안에서 도움을 준 모든 분들께 이자리를 빌어 작으나마 감사를 드립니다.

멀리서 꾸준히 디테일까지 신경 써준 이중록 선생, 인승균 선생, 이현숙 선생을 비롯한 피부과 동문 선배들에게도 고마움을 전합니다. 바빠서 자주 연락을 하지 못했던 ‘좋은 친구들’과 인하대 동문들에게도 미안함과 함께 감사함을 전합니다.

언제나 저를 위해 기도해주시고 물심양면으로 희생과 사랑을 아끼지 않았던 아버님과 어머님께 무한한 감사를 드립니다. 고맙습니다.

또한 제가 성장하고 발전하기까지 지켜봐 주셨던 장모님, 양가 친지 분들과도 기쁨을 함께 하고자 합니다.

결로 바쁘고 어려운 여간 속에서도 끊.Does not support ASCII characters yet. 관사과 격려를 해 준 아내과 두 아이에게 이 논문과 함께 깊은 감사를 전하고자 합니다.