Journal of Biochemistry and Molecular Biology, Vol. 39, No. 6, November 2006, pp. 662-670

JOURNAL OF
Biochemistry and Molecular Biology

Mistletoe Lectin (*Viscum album coloratum*) Modulates Proliferation and Cytokine Expressions in Murine Splenocytes

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Received 2 April 2006, Accepted 19 June 2006

It is well documented that an extract of European mistletoe has a variety of biological effects, such as the stimulation of cytokine production from immune cells, and additional immunoadjuvant activities. While the European mistletoe has been studied intensively, we know less about Korean mistletoe as a therapeutic plant, especially as a possible immunomodulating drug. This study will investigated the effects of Korean mistletoe lectin (*Viscum album* L. *var. coloratum* agglutinin, VCA) on murine splenocytes to investigate whether VCA acts as an immunomodulator, which could lead to improved immune responses in these cells. The results showed that VCA inhibited cell proliferation at higher concentrations (at 1-8 ng/ml) and enhanced cell proliferation at lower concentrations (at 4-32 pg/ml). Further studies were carried out to determine if the pro-proliferative or anti-proliferative activity exhibited by VCA was correlated with cytokine secretion. Consequently, interferon (IFN)-γ secretion was decreased in concanavalin A (ConA)-stimulated murine splenocytes by VCA (4-64 ng/ml), but there was no change in IL-4 levels. This suggests that VCA has the ability to modulate murine splenocyte proliferation and can possibly act on the balance of Th1/Th2 cellular immune responses.

Keywords: Cytokine, Immunomodulation, Lectin, Mistletoe, Proliferation

Introduction

Mistletoes belong to the families Loranthaceae and Viscaceae, which both are taxonomically related to each other. Scientific interest on mistletoe began in the 20th century, as Gaultier (Gaultier and Chevalier, 1907) investigated the hypotensive effect of oral or subcutaneous applications of fresh *Viscum album* L. extracts in man and in animals. The aqueous extract of European mistletoe (*Viscum album* L., Loranthaceae) has been used in conventional cancer therapy for decades, mainly in Europe (Büssing e*t al*., 1996). Recent scientific research has confirmed that mistletoe extract induced apoptotic killing of cultured human tumour cells and lymphocytes, and stimulated the immune system (Hajto *et al*., 1990; Schultze *et al*., 1991; Park *et al*., 2001; Urech *et al*., 2005). Many investigators have demonstrated that the biologically active components of European mistletoe are lectins, alkaloids, and polysaccharides (Khwaja *et al*., 1986; Hajto *et al*., 1989; Mueller and Anderer, 1990; Kuttan *et al*., 1992; Park *et al*., 1998a; Peng *et al*., 2005). D-galactose- and/or *N*-acetyl-D-galactosamine-specific lectins (*Viscum album* L. agglutinin, VAA-I, II, III) are considered to be major active components in European mistletoe and have molecular masses between 50 and 60 kDa (Franz *et al*., 1981). The VAAs are type-2 ribosome-inactivating proteins (RIPs) composed of two different subunits, an A- and B-chain linked by a disulfide bridge. The A-chain is capable of inactivating the 60S ribosomal subunit of eukaryotic cells resulting in inhibition of protein synthesis. The B-chain is capable of binding to cell-surface glycoconjugates and thereby permits entry into the cell of the toxic subunit (Peumans *et al*., 1996). Cell surface carbohydrate chains provide potential binding sites to endogenous carbohydrate binding proteins (Schumacher *et al*., 1994).

Semiparasitic plants, mistletoes, which are distributed in Korea and other East-Asian countries (mainly China and Japan) have long been recognized as therapeutic herbs (Li, 1975). It has been traditionally used as a sedative, analgesic, anti-spasmolytic, cardiotonic, and anticancer agent; the herbs are also used to tone the liver and kidneys, strengthen tendons and bones, expel pathogens associated with rheumatism,

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stabilize the fetus and cause lactogenesis. While the European mistletoe has been studied intensively, less is known about Korean mistletoe as a therapeutic herb, especially as a suggested anticancer drug. Various chemical components have been identified from the extracts of Korean mistletoe (*Viscum album* var. *coloratum*), such as lectin, steroid, triterpene, sesquiterpene lactone, carbohydrate, flavonoid, organic acid, alkaloid, amino acids, and peptides. A galactose- and *N*-acetyl-D-galactosamine-specific lectin (*Viscum album* L. var. *coloratum* agglutinin, VCA), which is known for its anti-cancer activity, was isolated from Korean mistletoe using SP Sephadex-C50, Sepharose 4B, and ultrafiltration (Lyu *et al*., 2000; Lyu *et al*., 2001; Lyu *et al*., 2002). Recent work suggested that Korean mistletoe is a potent immunoadjuvant, enhancing cellular and humoral immune responses (Yoon *et al*., 2001). It was also shown that the extract of Korean mistletoe had anti-tumour activity, inhibiting tumour metastasis in mice, and its anti-tumour activity was related to suppression of tumour growth and tumour-induced angiogenesis (Yoon *et al*., 1995), as well as enhancement of NK cell activity (Yoon *et al*., 1998). Furthermore, it is known that Korean mistletoe augments cellular and humoral immune responses such as antigen-specific antibody production and induction of cytolytic T lymphocyte (CTL) activity against tumour cells (Yoon *et al*., 1999).

Despite previous research, the potential leukocyte stimulatory effect of mistletoe lectin has not been studied in depth. In the present study, we were interested in obtaining proof of principle data in murine cells by: a) verifying if VCA induces proliferation in murine splenocytes in activated or non-activated conditions, and b) investigating whether VCA as an immunomodulator, which could lead to improved cell-mediated Th1/Th2 cytokine immune response in murine splenocytes. The parameters selected were INF-γ and IL-4. INF-γ is a cytokine which predominantly released by Th1 and NK cells, and which plays a major role in the cell-mediated immune response. IL-4 directs the development of Th2 cells both *in vitro* and *in vivo*, downregulates INF-γ production in Th1 cells (O'Garra and Murphy, 1993) and in human monocytes (Zurawski and de Vries, 1994b).

Materials and Methods

Purification of lectin. Korean mistletoe (*Viscum album* L. var. *coloratum*) growing on oak trees was collected in winter in Kangwon province, Korea. The botanical identity was established by Prof. Jon Suk Lee, College of Natural Sciences, Seoul Women's University. Crude protein was prepared by ion exchange chromatography (Park, 1998; Park, 1999b). Briefly, 3.5 g of SP Sephadex C-50 (Amersham Pharmacia Biotech. Inc.) was added to one liter of aqueous solution extracted from 100 g of air dried mistletoe and stirred at 4°C. The gel was then packed into a chromatography column and was washed with 0.1 M acetate buffer (pH 4.0), and bound protein eluted with 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl. The eluate was passed through a column packed with asialofetuin (Sigma)-Sepharose 4B and the column washed with phosphate-buffered saline (PBS, pH 7.0). Absorbed protein was eluted with 0.2 M acetic acid and concentrated by ultrafiltration (MW = 10 kDa, Amicon Corp., Danvers). Briefly, the membrane was placed into the holder, and the membrane holder was fitted into the cell body. The stirrer assembly was placed into the cell body, and the sample was poured and nitrogen gas was used to pressurize the cell. The cell was placed on the magnetic stirring table, and the stirring rate was adjusted until the vortex created was approximately one-third the depth of the liquid volume. The protein content was determined by protein assay (Bio-Rad Laboratories) using BSA (bovine serum albumin) as a standard. The mistletoe lectin was stored in −80°C until used. European mistletoe and the purified lectins (VAA-I, II, and III) were kindly provided by Prof. Pfüller (Institut für Phytochemie, Universität Witten/Herdecke).

Haemagglutination test. Mistletoe lectin activity was measured by haemagglutination titer. Haemagglutination test was performed by a serial two fold dilution method using 96 round-bottomed well plate (NUNC, Life Technologies) (Park, 1994; Park, 1995; Park, 1997; Park, 1998). Blood specimens were obtained with consent from healthy human volunteers (aged between 20 and 50 years). One milliliter of blood was collected into a pre-heparinised tube and the red blood cells immediately washed by adding 50 ml of 0.15 M NaCl and centrifuging at 600 × *g* for 5 min. The supernatant was discarded and the red blood cells were resuspended in 0.15 M NaCl to a 3% suspension. Fifty microliter of red blood cell suspension was added to 50 μl serially diluted VCA and the plate was incubated at 37°C for 1 hour. Results were shown as haemagglutinating unit (HU) which is defined as a maximum number of a serial two-fold dilution of lectin solution showing haemagglutinating activity.

SDS-PAGE. The molecular mass and purity of VCA was determined by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is a method for quantifying, comparing, and characterizing proteins. This method separates proteins based primarily on their molecular masses (Laemmli, 1970). SDS binds to hydrophobic portions of a protein, denatures secondary and non-disulfide-linked tertiary structures, allowing it to exist stably in the solution in an extended conformation. As a result, the length of the SDS-protein complex is proportional to its molecular weight. Twelve percent polyacrylamide (Sigma) gel was used as resolving gel and 4% was used as stacking gel. Resolving gel was allowed to polymerize for 30 minutes and stacking gel was allowed to polymerize for 10 minutes. To further denature the proteins by reducing disulfide linkages, the samples were heated at 98°C for 10 min in the presence of a reducing agent, 1% 2B-mercaptoethanol (Sigma). Bromophenol blue (0.1 mg/ml) was also added to the protein solution to serve as a tracking dye and the samples were loaded into the wells. The samples were electrophoresed using electrophoresis system (Bio-Rad Laboratories) at 100 V for 1 hour and stained with Coomassie brilliant blue R-250 for 1 h and destained with destaining solution.

Cell culture. Mouse splenocytes were maintained in complete cell culture medium (CTCM) consisting of RPMI 1640 (Sigma) supplemented with 5% fetal calf serum (FCS, Sigma), 100 U/ml penicillin/100 μg/ml streptomycin (Sigma), 2 mM L-glutamine

(Sigma), and 5×10^{-5} M 2-mercaptoethanol (2-ME). For cytokine production, cells (1×10^5 cells/well) were seeded in 96-well flat-bottomed tissue culture plates (NUNC, Life Technologies) in the absence or presence of 1 µg/ml concanavalin A (ConA, Sigma) and various VCA concentrations for different time intervals.

Cell density determination. Cell numbers and viability were assessed by trypan blue (Sigma) dye exclusion. The dye exclusion test is based on the concept that viable cells do not take up trypan blue dye, whereas dead cells are permeable to the dye. The cell suspension was gently swirled to distribute the cells evenly and 20 µl of trypan blue solution (0.4% (*w/v*) trypan blue in PBS) was mixed with 5 µl of cell suspension in a microtube. With a cover-slip in place, the trypan blue-cell suspension was transferred to the haemacytometer chamber carefully and continuously without overflow. The viable cells which did not take up trypan blue dye were counted in each of 25 squares in the large middle square. The cell concentration per ml and the total number of cells were determined using the following calculations.

Cells per ml = Total cell count in 25 squares $\times 10^4 \times 5$ (dilution factor)

Total cell number = Cells per ml × the original volume of fluid from which cell sample was removed

Assay of cytotoxicity. Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death. *In vitro* cell viability was measured using solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS, Promega) and an electron coupling reagent (phenozine methosulphate; PMS, Promega) which facilitates the reduction of MTS. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium (Barltrop, 1991; Cory, 1991; Riss, 1992). The MTS tetrazolium is similar to the widely used MTT tetrazolium, with the advantage that the formazan product of MTS reduction is soluble in cell culture medium. Metabolism in viable cells produces “reducing equivalents” such as NADH or NADPH. These reducing compounds pass their electrons to an intermediate electron transfer reagent that can reduce MTS into the aqueous, formazan product. Upon cell death, cells rapidly lose the ability to reduce tetrazolium products. The production of the colored formazan product, therefore, is proportional to the number of viable cells in culture. This technique is particularly useful for assaying cell suspensions because of its specificity for living cells. Briefly, cells were added in 96-well flat-bottomed tissue culture plates (NUNC, Life Technologies) in the absence or presence of VCA solution at various concentrations for 48 hours at 37°C in a humidified, 5% CO_2 atmosphere. At the end of the incubation, 40 µl of combined MTS/PMS solution was added into each well of the 96 well assay plate containing 100 µl of cells and 100 µl of samples in culture medium. The plate was incubated for 4 hours at 37°C in a humidified, 5% CO_2 atmosphere. The production of formazan was determined by measuring the absorbance of the compound at 450nm with a spectrophotometric 96-well plate reader (Dynex). The viability of the cells was calculated as: % viability = {(absorbance of treated cells)/ (absorbance of untreated cells)} × 100.

Proliferation of murine splenocytes stimulated with ConA. ConA cell proliferation assay was used to assess the effect of Korean mistletoe lectin on murine splenocyte activation and proliferation. Proliferation was assessed by the incorporation of [^{3}H]-thymidine into DNA. Eight-week-old female BALB/c mice were obtained from Harlan (Bicester) and given food and water *ad libitum*. Splenocyte suspensions were prepared by removing the spleens and placing them into RPMI 1640 medium (Sigma). The spleens were forced through 70-µm-pore-size wire gauzes using the plunger from a 5 ml syringe to produce a single cell suspension. The cells were pipetted into a sterile Universal tube and centrifuged at 600 g for 5 minutes. The supernatant was discarded and the cell pellet was gently tapped to resuspend. Two milliliter of sterile red cell lysis buffer (0.017 M Tris (Sigma), 0.144 M ammonium chloride buffer (Sigma), adjusted to pH 7.2) was added to the cells and was incubated for 5 minutes at room temperature. The cells were washed with RPMI 1640 medium with 2% (*v/v*) FCS and centrifuged at 600 g for 5 minutes. Residual red blood cells were lysed once more and washed twice as described above. The splenocytes were resuspended in 15 ml of CTCM, and the density and cell viability were determined using trypan blue assay. VCA compounds were tested at doubling dilutions ranging from 5 µg/ml to 100 pg/ml in a final volume of 200 µl CTCM, in the presence or absence of ConA (Sigma) at 1 µg/ml or 500 ng/ml and 100,000 splenocytes. The cells were incubated for 24 hours at 37°C in a humidified, 5% CO_2 atmosphere, after which 100 µl of culture supernatants was removed for the cytokine assays. Following further incubation for 24 hours, 0.25 µCi [^{3}H]-thymidine (Amersham Pharmacia Biotech. Inc.) in 10 µl volume made up in RPMI 1640 medium was added. The cells were incubated for further 24 hours at 37°C in a humidified, 5% CO_2 atmosphere, and were harvested onto 96-well filter plates (Unifilter Filtermate Harvester™, Packard Bioscience Ltd). Following the addition of 25 µl scintillant (MicroScint-O™, Packard Bioscience Ltd) to each well in the filter plates, the incorporated [^{3}H]-thymidine was measured with a β-scintillation counter (TopCount™, Packard Bioscience Ltd) according to manufacturer’s specifications. Results were expressed as the mean cpm of [^{3}H]-thymidine incorporated in triplicate cultures. The optimal dose of ConA at 1 µg/ml used in cell proliferation assays was determined from ConA titration curves (Fig. 1).

Proliferation of murine splenocytes stimulated with anti-murine CD3 antibody. The optimal dose of anti-CD3 antibody 3 µg/ml used in cell proliferation assays was determined from anti-human CD3/CD28 titration curves (Fig. 2). Cell suspensions of splenocytes from individual mice were prepared as described above. VCA compounds were tested at doubling dilutions ranging from 5 µg/ml to 100 pg/ml in a final volume of 200 µl CTCM, in the presence or absence of anti-CD3 antibody (BD Pharmingen, UK) at 1 µg/ml and 100,000 spleen cells. Murine splenocytes were incubated for 48 hours at 37°C in a humidified, 5% CO_2 atmosphere, followed by pulsing with 0.25 µCi [^{3}H]-thymidine (Amersham Pharmacia Biotech. Inc.) (see above). After a further incubation of 24 hours, cells were harvested onto 96-well filter plates (Unifilter Filtermate Harvester™, Packard Bioscience Ltd) and then counted as described previously.

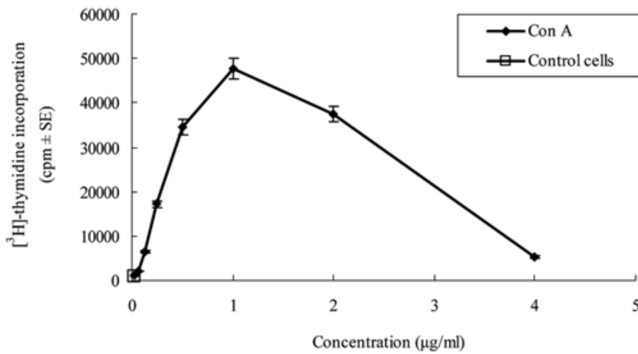

Fig. 1. The Concanavalin A (ConA) dose response in murine splenocytes. The optimal dose of ConA at 1 μg/ml used in cell proliferation assays was determined from these ConA titration curves.

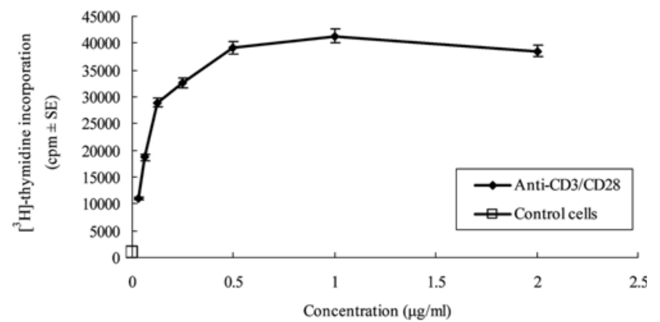

Fig. 2. The anti-human CD3/CD28 antibody dose response in human peripheral blood mononuclear cells. The optimal dose of anti-human CD3 antibody and anti-human CD28 antibody at 100 ng/ml and 5 μg/ml, respectively, used in cell proliferation assays was determined from anti-human CD3/CD28 titration curves.

INF-γ production from ConA and VCA stimulated murine splenocytes. Enzyme-linked immunosorbent assay (ELISA) was applied for qualitative and quantitative determinations of the cytokines in the culture supernatants. These tests were based on the sandwich principle. Recombinant murine cytokines were used as standards.

For determination of interferon-γ (INF-γ), 96-well Nunc MaxiSorp (Life Technologies) plates were coated with 2 μg/ml solution of 'capture' rat anti-mouse INF-ã monoclonal antibody (BD Pharmingen, UK) in 0.1 M carbonate/bicarbonate buffer, pH 9.0 overnight at 4°C. After washing the plates three times with PBS-Tween, which contained phosphate buffered saline (PBS) with 0.5% (*v/v*) Tween 20 (Sigma), the plates were blocked with 1% (*w/v*) bovine serum albumin (BSA) (Sigma) at room temperature for 2 hours. Following three washes with PBS-Tween, 50 μl of cell culture supernatants were added and incubated overnight at 4°C; standard mouse INF-γ concentrations (BD Pharmingen) ranging from 31.25 to 1,000 pg/ml were included for each plate. After four washes with PBS-Tween, the 'captured' INF-γ was sandwiched with 100 μl of biotinylated rat anti-mouse INF-γ monoclonal antibody (BD Pharmingen) which was diluted in 1% BSA in PBS-Tween, and incubated at room temperature for 1 hour. Following four washes, the presence of biotinylated antibodies was detected with 100 μl of a 1 : 1000 dilution of streptavidin-peroxidase (BD Pharmingen). At the end of an hour incubation at room temperature, the plates were thoroughly washed six times with PBS-Tween and the assay was developed using 100 μl of 0.1 mg/ml of 3,3',5,5'-tetramethylbenzidine substrate (Sigma) in 0.1 M sodium acetate buffer, pH 6.05, containing 0.03% hydrogen peroxide. The enzyme reaction was stopped with 50 L of 1 M hydrochloride after an incubation of 10 minutes at room temperature, and the colourimetric development was read at 450 nm with a spectrophotometric 96-well plate reader (Dynex Technologies). The concentrations of INF-γ in the culture supernatants were determined by extrapolation from the reference standard INF-γ curve.

IL-4 production from ConA and VCA stimulated murine splenocytes. The release of IL-4 was detected by sandwich ELISA, similarly described above for the INF-γ detection assay, using a 'capture' rat anti-mouse IL-4 monoclonal antibody (BD Pharmingen). Cell culture supernatants were added and incubated overnight at 4°C; standard mouse IL-4 concentrations (BD Pharmingen) ranging from 15.625 to 500 ng/ml were included for each plate. Biotinylated rat anti-mouse IL-4 monoclonal antibody (BD Pharmingen) was added and bound biotinylated antibody was detected with streptavidin-peroxidase. The assay was developed as previously described for INF-γ detection assay. The concentrations of IL-4 in the culture supernatants were determined by extrapolation from the reference standard IL-4 curve.

Statistical analysis for immunological data. A one-way ANOVA was used for multiple comparisons (MINITAB®, release 14.1, Minitab Inc.). For proliferation, MTS, and ELISA, Dunnett test was used to compare each of the treatment groups with control groups and the significance was determined. Probability values (*P*-value) of <0.001, <0.01 or <0.05 were considered significant with 99.9%, 99% or 95% of confidence, respectively.

Results

Purification of VCA. SP Sephadex C-50 is a strong cation exchanger and is used to separate proteins based on the charge. The ion exchange group is a sulphopropyl group which remains charged and maintains consistently high capacity over the entire working range of pH 4-13. Korean mistletoe lectin was isolated by purification through SP Sephadex C-50 and then on asialofetuin-Sepharose 4B. Compared with the results reported (Park *et al.*, 1997; Park *et al.*, 1998b), lectin was efficiently isolated by the asialofetuin-immobilized chromatography.

Haemagglutination test. Korean mistletoe lectin isolated by asialofetuin-immobilized Sepharose 4B had no blood group specificities for human blood type A, B, O, and AB. The lectin (1 μg/ml) agglutinated the erythrocytes tested, showing haemagglutinating activity of 128 HU.

SDS-PAGE. In the absence of the reducing agent (2B-

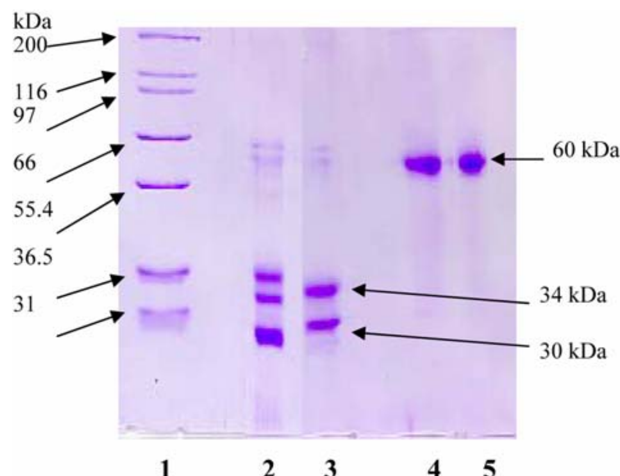

Fig. 3. SDS-PAGE profiles of VCA in the absence or presence of reducing agent. VCA isolated by SP-50 Sephadex C-50 and asialofetuin-Sepharose 4B were determined by SDS-PAGE in the presence (lane 2) and in the absence (lane 3) of reducing agent. Lane 1; molecular weight marker, lane 2, 3; VCA.

mercaptoethanol), the molecular mass of Korean mistletoe lectin (VCA) was 60 kDa which is similar to the molecular mass of European mistletoe lectin (VAA-II) (Fig. 3) (Lyu *et al.*, 2000). On the other hand, in the presence of the reducing agent, Korean mistletoe lectin (VCA) showed two bands consisting of a 30 kDa A-chain and a 34 kDa B-chain (Fig. 3), whereas European mistletoe lectin (VAA) shows three bands (36 kDa, 33 kDa, and 29 kDa) (Franz *et al.*, 1981, Lyu *et al.*, 2000). These patterns indicate that only one lectin is isolated from Korean mistletoe by asialofetuin-Sepharose 4B.

Cytotoxic activities. In order to discern whether inhibition of proliferation was due to cytostatic or cytotoxic effect, and in order to verify that the administration dose had minimal effect on the cells, the viability of mouse splenocytes was measured 48 h post-treatment. The cells were treated for 48 hours with VCA or VCA + anti-CD3 antibody at the indicated dose and the viability was measured using the MTS assay. Mouse splenocytes showed more than 90% of the cells survived at concentrations lower than 8.4 ng/ml, which is the highest concentration used in proliferation assay (Fig. 4). The result obtained showed that the selected concentration of VCA had no major effect on the cells when compared to the controls.

Effects of VCA on murine splenocytes proliferation. Murine spleen cells were used to evaluate the immunomodulatory activity of VCA. Splenocytes were stimulated with the T cell mitogen, ConA or anti-CD3 antibody for 48 h. The results showed that when 1 pg/ml-8 ng/ml of VCA was used, ConA-stimulated splenocytes proliferation was inhibited by the VCA at high concentrations (1-8 ng/ml). On the contrary, VCA at low concentrations (4-32 pg/ml) stimulated murine splenocyte proliferation, while non-stimulated splenocytes were not affected (Fig. 5). Anti-CD3 antibody-stimulated splenocytes showed similar results (inhibition at 1-8 ng/ml, stimulation at 4-16 pg/ml) (Fig. 6) indicating that VCA effect in the presence of mitogen ConA, also a lectin, is not likely to be due to a competitive effect.

Murine splenocyte cytokine secretion following stimulation with ConA. The stimulation of murine splenocytes with VCA alone did not cause any significant INF-γ release in a concentration range of 0.05-64 ng/ml (Fig. 7). However, in

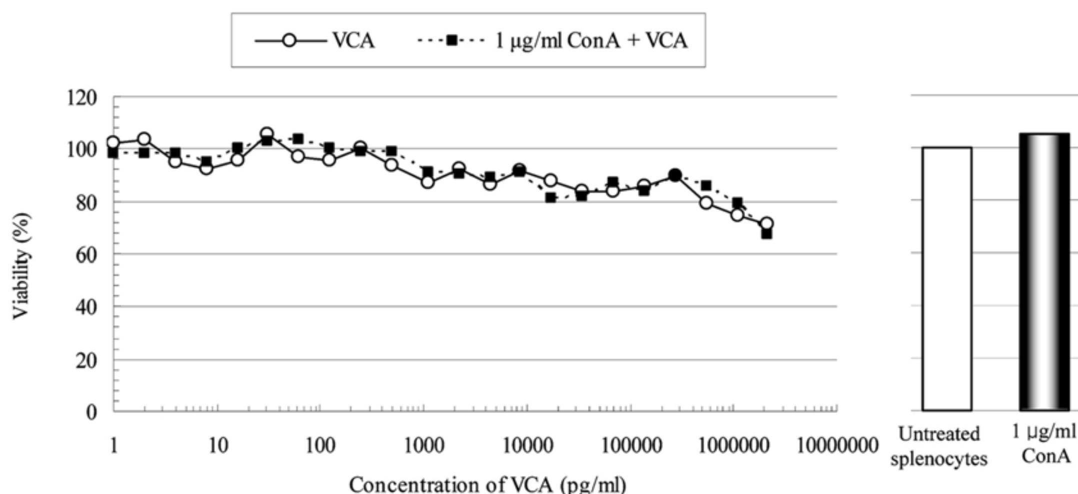

Fig. 4. Murine splenocytes were cultured in absence or presence of different concentrations of VCA and the viability was measured by MTS assay. The viability was also measured when the cells were treated with or without ConA (1 μg/ml). Mouse spelnocytes were cultured in absence or presence of different concentrations of VCA and the inviability was measured by MTS assay. Mouse splenocytes showed more than 90% of the cells survived at concentrations lower than 8.4 ng/ml. The result obtained showed that the selected concentration of VCA had no major effect on murine splenocytes when compared to the control cells. Experiments were performed four times with similar results and each point represents the mean of triplicates.

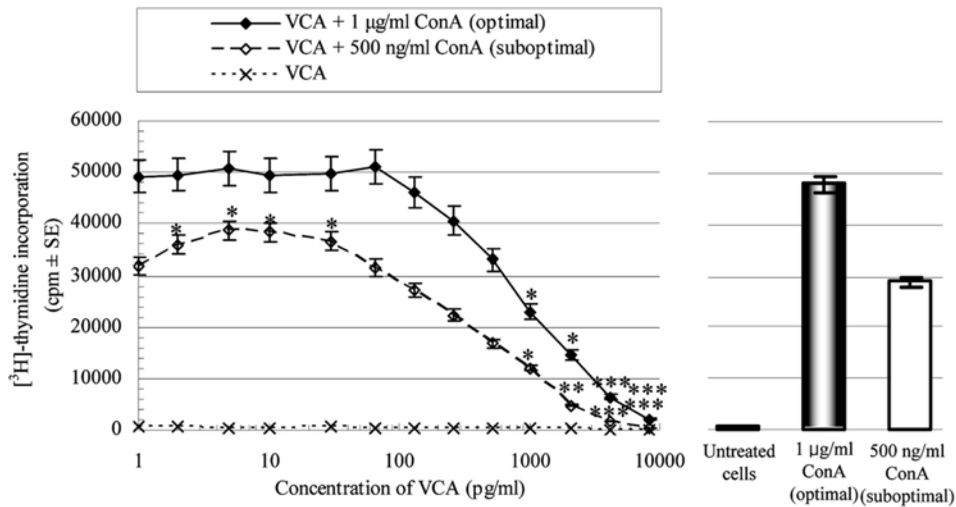

Fig. 5. The effect of VCA on ConA-stimulated murine splenocyte proliferation. Splenocytes were stimulated with an optimal ConA concentration (1 μg/ml) or sub-optimal ConA concentration (500 ng/ml). VCA inhibited ConA-stimulated cell proliferation at high concentrations but stimulated the proliferation at low concentrations. No significant effect was shown when cells were treated with VCA alone. Results correspond to the mean ± SE of more than five independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

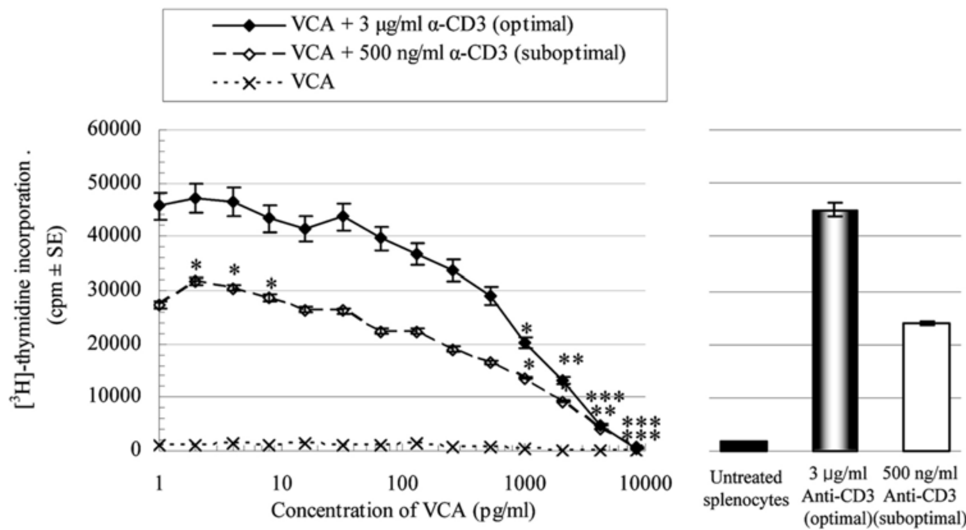

Fig. 6. The effect of VCA on anti-CD3 antibody-stimulated murine splenocyte proliferation. Splenocytes were stimulated with an optimal (3 μg/ml) and sub-optimal anti-CD3 antibody (500 ng/ml) concentration. VCA inhibited anti-CD3 antibody-stimulated cell proliferation at high concentrations but stimulated the proliferation at low concentrations. No significant effect was shown when cells were treated with VCA alone. Results correspond to the mean ± SE of more than five independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

ConA-stimulated splenocytes, 4-64 ng/ml VCA shifted the ConA-induced INF-γ secretion toward a more immunosuppressive response. In the concentration range of 0.05-2 ng/ml VCA, there was no significant change compared with ConA control (Fig. 7). Overall, the levels of INF-γ released from ConA stimulated murine splenocytes in the presence of VCA revealed a similar pattern to cellular proliferation. The control (CTCM) did not exert any effect on the ConA-stimulated release of INF-γ. There were no significant changes in IL-4 release (data not shown).

Discussion

Korean mistletoe, a subspecies of European mistletoe, was previously isolated from extracts of *Viscum album* L. *var. coloratum*, and its biochemical properties were characterized. Among the mistletoe components, the cytotoxic and immunological properties of mistletoe preparation are considered to be linked to lectins which are focus of modern biomedical research. The sugar-binding B chain of VAA-I is able to bind galactoside residues on the cell membrane

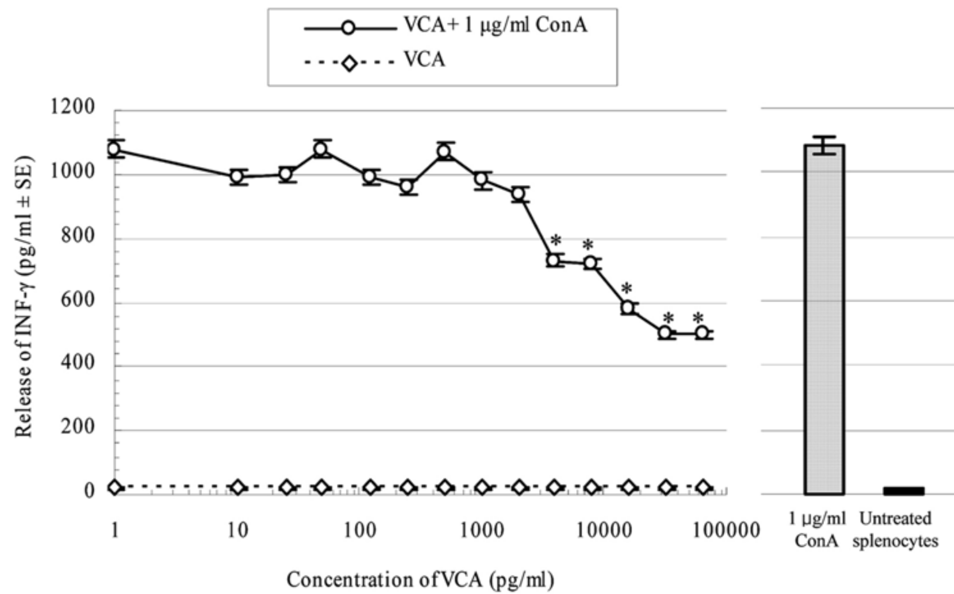

Fig. 7. The effect of VCA on INF-γ secretion in murine splenocytes costimulated with ConA. Murine splenocytes in RPMI 1640 (5%) was stimulated with 1 μg/ml ConA in the presence of the indicated concentrations of VCA. After 48 hr of incubation at 37°C, released cytokines were measured in the cell-free supernatants by ELISA. As a result, INF-gamma secretion was decreased in ConA-stimulated murine splenocytes by VCA (4-64 ng/ml). Results correspond to the mean ± SE of more than five independent experiments. $*p < 0.05$.

preferring certain confirmations (Hostanska *et al*., 1995). In low (not cytotoxic) doses, the B chain is responsible for the enhancing effect of VAA-I on the proinflammatory activity of natural immune system. After 24 h incubation with human PBMC, VAA-I (A and B chains in nanogram per milliliter concentrations) induced mRNA expression and enhanced secretion of proinflammatory cytokines (Hajto *et al.*, 1990; Hostanska *et al.*, 1995). In several *in vitro* and *in vivo* studies, activation of NK-cells, monocytes/macrophages, T-cells, especially T-helper cells, cytokine release, induction of apoptosis, protection against sister chromatid exchange inducing DNA damage were demonstrated (Büssing, 1997), effects which are believed to be beneficial for tumour defense. Modulation of the immune response may be manifested in various ways. Immunosuppression may lead to an enhanced host susceptibility to infectious or neoplastic disease, whereas immunostimulation (immunoenhancement) may ultimately result in hyperimmune conditions including allergy or autoimmune disease (Gleichmann *et al*., 1989).

In the present study, immune response parameters were evaluated to determine the potential immunomodulatory effect of VCA. To understand this, first the direct effect of VCA on lymphocyte proliferation was investigated. In the *in vitro* assays, murine splenocyte proliferation was not affected. In order to demonstrate if the anti-proliferative activity was associated with cytotoxicity of VCA, MTS/PMS solution was incubated with the cells and MTS assay was performed. The result obtained showed that the selected concentration of VCA had no major effect on the cells when compared to the controls (Fig. 4). It has been reported that VCA has a strong killing effect on colon cancer cells and A253 (head and neck cancer) cells, but no effect on normal cells (Choi *et al.*, 2004). These results indicate that VCA treatment selectively kills cancer cells, but not normal cells. The reason for the selective cell death is unclear, but one may suggest that differences in the mistletoe lectin surface affinity and/or intracellular uptake of the toxic proteins, and their subsequent degradation could be determinating factors.

The next step was to investigate whether the association between VCA and stimulants affected the proliferation. In mouse splenocytes, at high VCA concentrations, ConA polyclonal activation was decreased. In order to discard the possibility of killing cells due to an excessive activation, a sub-mitogenic concentration of ConA (500 ng/ml) was used. The same kinetics was observed and high VCA concentrations inhibited the splenocyte proliferation. However at low VCA concentrations, VCA displayed a proliferating activity than those of cells stimulated with ConA alone (Fig. 5). This shows that VCA can act *in vitro* on the regulation of cellular immunity inducing proliferation of lymphocytes.

Furthermore, as VCA is galactose- and *N*-acetyl-D-galactosamine-specific on the cell membrane, anti-CD3 activated splenocyte was used to confirm that the inhibition of proliferation isn't competitive with ConA. *In vitro* T lymphocyte stimulation is used extensively to facilitate T cell expansion and in the study of T cell function. Mitogenic molecules such as lectins and monoclonal antibodies promote polyclonal proliferation whereas specific antigen yields a monoclonal or oligoclonal response. Phytohemagglutinin (PHA) and concanavalin A activate T cells by binding to cell

membrane glycoproteins, including the T cell receptor (TCR)-CD3 complex (Kay *et al*., 1991). The anti-CD3 can be used to stimulate T cells in assays, for this method has several advantages over the ConA assay, including the ability to specifically analyze T cell proliferation. As a result, VCA showed similar effect on anti-CD3 activated splenocytes as ConA-splenocytes, proving that VCA is directly suppressing T-cell proliferation (Fig. 6).

In addition, we investigated on whether VCA as an immunomodulator could lead to increased cytokine production associated with cell-mediated immunity. The parameters selected were INF-γ and IL-4 in murine splenocytes. INF-ã is a cytokine which predominantly released by Th1 and NK cells, and which plays a major role in the cell-mediated immune response. IL-4 directs the development of Th2 cells both *in vitro* and *in vivo*, downregulates INF-γ production in Th1 cells (O'Garra *et al*., 1993) and in human monocytes (Zurawski and de Vries, 1994a). After incubation of splenocytes with VCA, cytokine induction was measured at a comparable lectin concentration range. INF-γ secretion into the supernatants of the cells was dose-dependently inhibited by VCA (Fig. 7), while IL-4 was not detected at all. This might be due to a different binding affinity which is related to a different cytokine-inducing effect. There are previous reports indicating that that mistletoe lectin binds to much higher degree to monocytes than lymphocytes (Hostanska *et al*., 1995; Hajto *et al*., 1997).

In conclusion, results showed that VCA has a dual effect on proliferation in immune cells (inhibits at higher concentration and induces at lower concentration). This suggests that shows VCA has the ability to modulate murine splenocytes and can act on the regulation of cellular immune responses *in vitro*. In addition, INF-γ and IL-2 secretion into the supernatants of the cells were dose-dependently increased by VCA, but there were no change in IL-4 secretion. The correct judgment as to whether the proliferation is a direct effect of the lectin on lymphocytes or it is a consequence of the lectin-induced proinflammatory cytokines has yet to be determined. Furthermore, in previous reports, VCA showed strong cytotoxic effects on various cancer cells (*i.e.* lung, liver, melanoma, breast, head and neck) (Choi *et al.*, 2004). However in this study, VCA had little or no cytotoxicity in murine splenocytes using the same immunomodulating concentrations, which implies that VCA has a selectivity of killing cells. The reason for the selective cell death is unclear, but one may suggest that differences in the mistletoe lectin surface affinity and/or intracellular uptake of the toxic proteins, and their subsequent degradation could be determinating factors. Taken together, a) VCA inhibits proliferation in stimulated murine splenocytes at high concentrations (1-8 ng/ml), and induces in low concentrations (4-32 pg/ml) and b) VCA can inhibit INF-γ in high concentrations (4-8 ng/ml), but does not affect the production of IL-4 cytokine in murine splenocytes. These data indicate that VCA has the ability to modulate the murine splenocyte immune status and can act on the regulation of cellular immune responses *in vitro*. This report is aimed at providing the mistletoe research community with a robust data base on which further studies could be built.

Acknowledgment This study was supported by research grant of Seoul Women's University in the program year of 2005.

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