Article

Immunomodulatory Effects of Esculetin (6,7-Dihydroxycoumarin) on Murine Lymphocytes and Peritoneal Macrophages

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Coumarins belong to a diverse group of naturally occurring non-nutrient phytochemicals known as benzo-α-pyrones. In this study, esculetin, a 6,7-dihydroxy derivative of coumarin with pleiotropic biological activities, was found to have no significant cytotoxic effect on normal murine macrophages, but it could increase the *in vivo* migration of the thioglycollate-elicited macrophages in a dose-dependent manner. Moreover, esculetin significantly increased the endocytic activity, and augmented the nitric oxide production and *iNOS* gene expression in LPS-treated macrophages. In addition, *in vivo* administration of esculetin into mice was shown to increase the mitogenesis of splenic lymphocytes towards Con A and LPS stimulations, and induced the LAK activity of splenic lymphocytes. Collectively, our results indicate that esculetin could exert immunomodulatory effects on murine macrophages and lymphocytes, both *in vitro* and *in vivo*, and this might be one of the possible mechanisms by which coumarins can exert their chemopreventive and anti-tumor activities *in vivo*. *Cellular & Molecular Immunology*. 2005;2(3):181-188.

Key Words: immunomodulatory, esculetin, coumarin, murine macrophage, LAK cell

Introduction

In recent years the medicinal values of phytochemicals have aroused much concern, especially as immunomodulators or agents used for the adjunctive treatment of cancer. Coumarins belong to a diverse group of naturally occurring non-nutrient phytochemicals known as benzo-α-pyrones. They are commonly found in foodstuffs such as soybeans and cruciferous vegetables, and are widely used in perfumes, cosmetics, and as a flavor-enhancing agent for many different types of foods (1). Research in the past two decades has shown that coumarins possess diverse biological and pharmacological properties, such as anti-edema, anti-inflammatory, anti-carcinogenic, immunomodulatory and anti-tumor activities (2-4). Coumarins had undergone clinical trials for the treatment of cancer (5), lymphedema (6), chronic infections and disorders of the immune system (7). Esculetin (6,7-dihydroxycoumarin) is a coumarin derivative that can be isolated from many plants such as *Artemisia capillaries*, *Citrus limonia* and *Euphorbia lathyris* (8, 9). It is known to possess pleiotropic biological activities including lipoxygenase-inhibitory activity (10, 11), free radical scavenging activity (8, 12), suppressive activity on oxidative damage to DNA (13, 14), tyrosinase-inhibitory activity (9) as well as cancer chemo-preventive (15, 16) and anti-tumor activities (17-19).

Some earlier studies had demonstrated that coumarins could exhibit an immuno-enhancing effect on macrophages. For example, coumarins were found to increase the proteolytic capacity of macrophages (20) and stimulate the phagocytic activity of murine peritoneal macrophages (21). However, there are also reports showing that some coumarin derivatives exhibited no significant effect or displayed an inhibitory effect on macrophage functions. These include the inability of 7-amino-4-methylcoumarin to influence the phagocytosis of immune complexes by macrophages (22). Moreover, 4-hydroxy- and 7-hydroxycoumarins neither induced cytotoxic macrophages against P-815 tumor cells nor increased the release of tumor necrosis factor-α (TNF-α) from macrophages (23). On the other hand, cloricromene, a semi-synthetic coumarin derivative, inhibited the lipopolysaccharide (LPS)-

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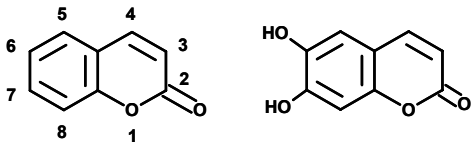
Abbreviations: Con A, concanavalin A; DMSO, dimethyl sulfoxide; iNOS, inducible nitric oxide synthase; IL-2, interleukin-2; *i.p.*, intraperitoneally; LAK, lymphokine-activated killer; LPS, lipopolysaccharide; NO, nitric oxide; PEC, peritoneal exudate cell; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α.



Coumarin **Esculetin**

Figure 1. Chemical structures of coumarin and esculetin (6,7-dihydroxycoumarin).

induced TNF-α production in rat macrophages (24). Although monocytes/macrophages appear to be an important target for the immunomodulatory action of coumarins, yet their modulatory effects on other immunocytes, and their action mechanisms and relationship to their anti-tumor activity remain poorly understood. In this paper, the immunomodulatory effects of esculetin, a 6,7-dihydroxy derivative of coumarin, on the murine peritoneal macrophages, lymphocyte mitogenic responses and lymphokine-activated killer (LAK) cell activity were investigated.

Materials and Methods

Mice

Inbred BALB/c mice were bred under a specific pathogen-free condition at the University Laboratory Animal Services Center of The Chinese University of Hong Kong. Mice of the same age (6-10 weeks old) and same sex were used in each experiment.

Chemicals

Esculetin (6,7-hydroxycoumarin) with an estimated purity of 98% was purchased from Aldrich Chemical Co. The chemical structures of esculetin and its parental compound, coumarin, are depicted in Figure 1. Esculetin was prepared as a 1 M stock solution in dimethyl sulfoxide (DMSO) and stored in dark at -20°C until use. Thioglycollate powder was purchased from Difco Lab and mitogens such as concanavalin A (Con A) and LPS were obtained from Sigma Chemical Co.

In vivo macrophage migration assay

The method described by Gervais et al. (25) was adopted. Briefly, BALB/c mice in groups of five were injected intraperitoneally (*i.p.*) on day 0 with 0.5 ml esculetin or the control DMSO solvent in diluted plain RPMI medium. On day 2, they were injected with 1 ml 3% thioglycollate broth. After 3 days, the peritoneal exudate cells (PECs) were harvested and cells recoverable from the peritoneal cavity were counted by trypan blue exclusion assay. The number of peritoneal macrophages was enumerated by determining the number of PECs that adhered to plastic surface.

Study of endocytic activity of macrophages

BALB/c mice were each injected *i.p.* with 1 ml 3% thioglycollate solution to elicit the peritoneal macrophages. Three days later, peritoneal cells were harvested by washing out the cells three times with plain RPMI medium. The PECs were seeded in a 24-well plate at 2.5×10^6/ml for 3 h to allow the adherence of macrophages to the bottom of the wells. The non-adherent cells were removed and different concentrations of esculetin were added with 20 ng/ml LPS. They were incubated at 37°C in a humidified CO_2 incubator for 48 h. The medium was removed and washed with warm plain medium. The cells were replenished with fresh assay medium together with 0.1 ml FITC-conjugated albumin (1 mg/ml) and were incubated at 37°C in dark for 6 h. After incubation, the cells were removed from the plate by pipetting up and down and were washed three times with cold plain medium. The pellet was resuspended in 1 ml 1% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). At least 10,000 cells for each sample were analyzed by flow cytometry using a FACSort flow cytometer (Becton Dickinson).

Determination of nitric oxide (NO) production by macrophages

Thioglycollate-elicited mouse peritoneal exudate cells (2.5×10^6/ml) were seeded in a 96-well flat-bottomed microtiter plate for 3 h to allow the macrophages to adhere to the bottom of the wells. Then non-adherent cells were removed and different concentrations of esculetin were added with or without 20 ng/ml LPS. They were then incubated at 37°C in a humidified CO_2 incubator for a defined period of time. After incubation, 100 μl of cell-free supernatant from each well was transferred into another 96-well flat-bottomed microtiter plate. Then 100 μl of freshly prepared Griess reagent was added onto each well and the plate was incubated at room temperature for 10 min with shaking. Finally, the absorbance at 540 nm was read by a Benchmark microplate reader (BioRad). The nitrite concentrations of the samples were quantified with reference to a series of standard sodium nitrite solution ranging from 0 to 64 μM.

Detection of inducible nitric oxide synthase (iNOS) gene expression by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared from 10^7 esculetin-treated peritoneal macrophages using the TRIZOL reagent (Gibco BRL). Specific amplification of murine *iNOS* gene using RT-PCR was performed as described previously (26). Briefly, 2 μg RNA was reverse transcribed in a 20 μl reaction mixture containing 200 U of M-MLV reverse transcriptase (Gibco BRL), 40 U of $RN_{ASE}OUT^{TM}$ recombinant ribonuclease inhibitor (Gibco BRL), 0.5 mM of each dNTP (Pharmacia Biotech.), 0.1 μg oligo$(dT)_{12\text{-}18}$ (Promega), 1× M-MLV first strand buffer and 10 mM dithiothreitol (Gibco BRL). A negative control without RNA samples was used to check for any contaminations. The reaction mixture was incubated at 37°C for 1 h, followed by 99°C for 5 min to inactivate the reverse transcriptase and to completely denature the template, and then the samples were cooled down to 4°C. The resulting cDNA sample was stored at -20°C until use. PCR was performed using the following synthetic pairs of primers supplied by Gibco BRL: *iNOS* sense strand: CTG CAT GGA CCA GTA TAA GGC AAG; *iNOS* anti-sense strand: GGG ACA GCT TCT GGT CGA

TGT CAT GA; *GAPDH* sense strand: AAT GGT GAA GGT CGG TGT GAA C; *GAPDH* anti-sense strand: GAA GAT GGT GAT GGG CTT CC. Two microlitres of each cDNA sample (equivalent to 0.1 μg of total RNA) was mixed with a 23 μl reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of Thermoprime^{Plus} DNA polymerase (Advanced Biotechnologies), and 0.2 μM of each sense and anti-sense oligonucleotide primer in a PCR tube. A negative control without cDNA sample was prepared to check for any contaminations. The reactions were performed in a thermocycler, GeneAmp PCR System 9700 (Perkin-Elmer Co.). Briefly, the samples were firstly subjected to an initial denaturation at 94°C for 5 min. Then 20-30 thermal cycles were carried out. The thermal cycle basically consisted of denaturation at 94°C for 30 sec, annealing at 56°C to 61°C for 60 to 75 sec and elongation at 72°C for 60 sec. In the last cycle, one more step in elongation for 5 min was performed to complete the reaction. Then the samples were cooled down to 4°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel at a constant voltage of 100 volts for about 1.5 h, stained with 1 μg/ml ethidium bromide solution and visualized and analyzed by the Bio-Rad Gel Doc 2000 under UV illumination. The gel images were captured and the intensity of each gel band was quantified by the ImageQuant software (Molecular Dynamics). The relative intensity of each band was compared with the untreated control, after normalization with respect to the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Mitogen-induced lymphoproliferative response

BALB/c mice in groups of five were injected *i.p.* with solvent control, 2 mg/kg or 8 mg/kg esculetin. Three days later, mouse spleen cells were cultured in 96-well flat-bottomed microtiter plates at 5×10^5 per well in the absence or presence of different concentrations of mitogens (LPS or Con A) at 37°C for 48 h inside a humidified 5% CO_2 incubator. The cells were pulsed with 0.5 μCi ^{3}H-thymidine (^{3}H-TdR) in 20 μl complete medium for 8 h. The cells were harvested onto a glass microfiber filter, and the ^{3}H-TdR incorporation was measured by the liquid scintillation counter (Beckman LS 2900 TR). The degree of mitogenesis was expressed as Stimulation Index (SI) which represented the fold increase in the proliferation of treated cells as compared to the untreated spleen cells obtained from the solvent control group of mice.

Measurement of lymphokine-activated killer (LAK) cell activity

LAK cell activity was measured by a colorimetric assay as described previously (27). Briefly, BALB/c mice in groups of five were injected *i.p.* with solvent control, 2 mg/kg or 8 mg/kg esculetin. Three days later, mouse spleen cells were prepared and cultured in 60 mm petri-dish at a cell density of 2.5×10^6 cells/ml in the absence or presence of 50 U recombinant murine interleukin-2 (IL-2) (Pepro Tech, specific activity was 5×10^6 U/mg) at 37°C in a humidified 5% CO_2 incubator. After 3 days of incubation, *via*ble spleen cells collected after centrifugation on Ficoll-paque gradient were added (7.5×10^5 cells/well) into the wells of a 96-well flat-bottomed microtiter plate which were pre-seeded overnight with 3×10^4/well of the mouse fibrosarcoma WEHI-164 target cells. The cell mixtures were incubated for 24 h at 37°C. The LAK activity was analyzed using the neutral red assay as described previously (27). The LAK activity was expressed as the % cytotoxicity on WEHI-164 cells which was calculated as follows: % cytotoxicity on WEHI-164 cells = (C – T) / C × 100%, where C = OD_{540} of control (without effector cells) and T = OD_{540} of test culture (with effector cells).

Table 1. Effect of esculetin on the *in vivo* migration of macrophages

Treatment of mice	Number of peritoneal macrophages recovered from each mouse ($\times 10^6$)
Experiment I	
Solvent control	3.36 ± 0.72
Esculetin (2 mg/kg)	2.31 ± 0.52
Experiment II	
Thioglycollate	8.17 ± 0.55
Thioglycollate + esculetin (2 mg/kg)	11.83 ± 0.74*
Thioglycollate + esculetin (4 mg/kg)	13.19 ± 0.51*
Thioglycollate + esculetin (8 mg/kg)	13.18 ± 2.01**

In Experiment I, BALB/c mice in groups of five were injected *i.p.* with solvent control (DMSO) or esculetin (2 mg/kg). Three days later, the PECs were harvested by peritoneal lavage and the numbers of peritoneal macrophages were determined. In Experiment II, BALB/c mice in groups of five were injected *i.p.* with solvent control (DMSO) or different doses of esculetin (2 mg/kg, 4 mg/kg and 8 mg/kg) on day 0. On day 2, mice were injected *i.p.* with 1 ml 3% thioglycollate broth. Three days later, the numbers of peritoneal macrophages were determined. The results were expressed as mean ± SE. * Significantly different from the thioglycollate group, $p < 0.01$. ** Significantly different from the thioglycollate group, $p < 0.05$.

Statistical analysis

All results were expressed as the arithmetic mean ± standard error (SE) of triplicate or quadruplicate determinations performed under the same conditions. The differences between the control group and treatment groups were determined by the Student's *t*-test and $p < 0.05$ was regarded as significantly different. The data shown were representative of two to three independent experiments.

Results

Effect of coumarins on the in vivo migration of macrophages

It has been known that *i.p.* injection of irritant substances, such as thioglycollate broth, can increase the yield of macrophages in the peritoneal cavity of mice (28). Therefore, the effect of esculetin on the *in vivo* migration of macrophages was studied. As shown in Table 1, injection of esculetin alone did not induce an influx of macrophages into the peritoneal cavity of mice. However, esculetin could

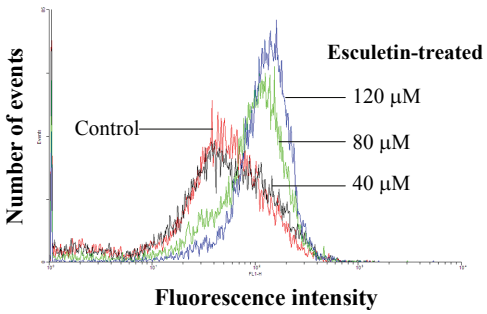


Figure 2. Effect of esculetin on the endocytic activity of LPS-treated murine macrophages *in vitro*. Thioglycollate-elicited murine macrophages were incubated either with solvent control or with different concentrations (40 μM, 80 μM and 120 μM) of esculetin in the presence of LPS (20 ng/ml) for 2 days at 37°C. Endocytic activity of macrophages was measured by their ability to engulf the FITC-conjugated albumin and the fluorescence uptake was analyzed by flow cytometry.

significantly increase the influx of macrophages elicited by thioglycollate in a dose-dependent manner. Using the colorimetric MTT assay, esculetin was found to exhibit no significant cytotoxic effect on the peritoneal macrophages *in vitro* at concentrations up to 500 μM after 48 h of incubation at 37°C (data not shown).

Effect of esculetin on the endocytic ability of murine macrophages

The ability of esculetin to modulate the endocytic activity of macrophages was examined. Endocytic activity of macrophages was measured by their ability to engulf the FITC-conjugated albumin and the fluorescence uptake was analyzed by flow cytometry. Figure 2 shows that esculetin at 40 μM concentration had no effect on the LPS-induced

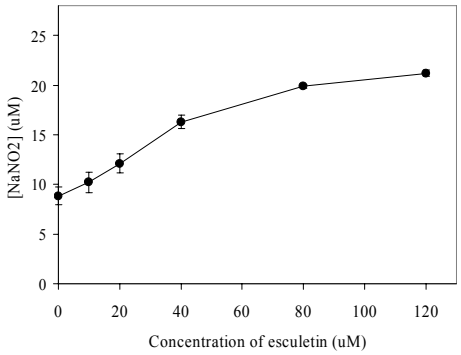


Figure 3. Effect of esculetin on the NO production of murine macrophages in the presence of LPS *in vitro*. Thioglycollate-elicited murine macrophages were incubated with different concentrations of esculetin (0-120 μM) in the presence of 20 ng/ml LPS for 2 days at 37°C. The amount of NO generation was detected by the Griess reagent. The results were expressed as mean ± SE of quadruplicate cultures.

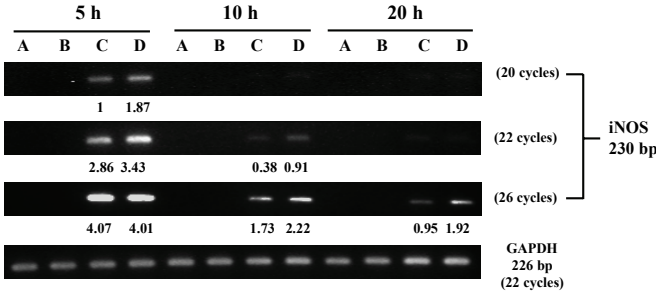


Figure 4. Effect of esculetin on the *iNOS* gene expression in murine macrophages. Thioglycollate-elicited murine macrophages (10^7) were either untreated or treated with 80 μM esculetin at 37°C for different time intervals (5, 10 and 20 h) in the presence or absence of 20 ng/ml LPS. Total RNAs were extracted and converted into cDNA by reverse transcription, and the target genes were amplified using specific primer pairs. The PCR products were separated on 2% agarose gels and their amounts were quantified by ImageQuant software. The value below each band in Lane D represents the relative intensity when compared with the group treated with LPS only (Lane C), after normalization with respect to GAPDH. Lane A, untreated macrophages; Lane B: macrophages treated with 80 μM esculetin; Lane C, macrophages treated with 20 ng/ml LPS; Lane D, macrophages treated with both esculetin (80 μM) and LPS (20 ng/ml).

endocytic activity of the macrophages. However, esculetin at higher concentrations such as 80 μM and 120 μM could enhance the endocytic activity of LPS-treated macrophages.

Effect of esculetin on nitric oxide production and iNOS gene expression in murine macrophages

Since nitric oxide has been shown to play an important cytotoxic role in the host defense against invading microorganisms and neoplastic cells (29), therefore, both the NO production and the *iNOS* gene expression in esculetin-treated macrophages were investigated. The ability of esculetin to modulate NO production by macrophages was measured using the Griess reagent while the modulatory effect of esculetin on *iNOS* gene expression was measured by the semi-quantitative RT-PCR technique. Our results showed that esculetin alone was unable to induce NO production by macrophages in the absence of LPS *in vitro* (data not shown). However, esculetin synergistically enhanced the LPS-induced NO production by macrophages in a dose-dependent manner (Figure 3). Moreover, the expression of *iNOS* gene was also examined in the esculetin-treated macrophages in the absence or presence of LPS. It was found that the esculetin alone was unable to induce the *iNOS* gene expression; however, it was capable of augmenting the *iNOS* gene expression in macrophages stimulated with LPS (Figure 4).

Effect of esculetin on the lymphoproliferative response of murine spleen cells

BALB/c mice were injected *i.p.* with two different doses of esculetin (2 mg/kg or 8 mg/kg) three days before their spleen cells were harvested and the lymphoproliferative response

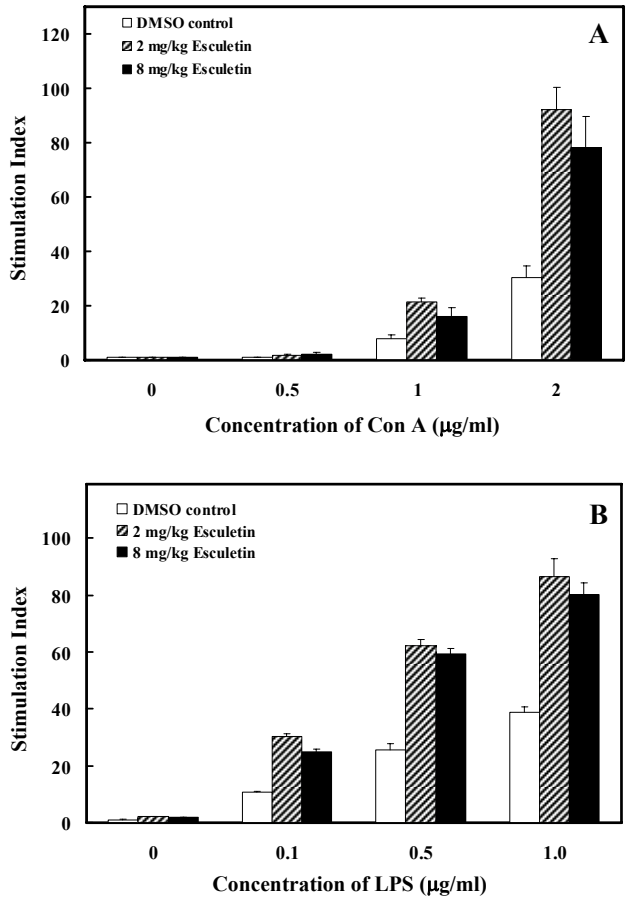


Figure 5. Esculetin enhanced the lymphoproliferative responses induced by Con A and LPS. BALB/c mice in groups of five were either injected *i.p.* with solvent control or esculetin (2 mg/kg and 8 mg/kg). Three days later, spleen cells were harvested and incubated with different concentrations of Con A (A) or LPS (B) at 37°C for 48 h at a cell density of 2.5×10^6 cells/ml. ^{3}H-TdR incorporation assay was performed. The results were expressed as stimulation index (SI) which represented the fold increase in the cell proliferation of the treated cells as compared to the untreated spleen cells (no *in vitro* mitogen treatment). The data shown are the mean ± SE of quadruplicate cultures.

was measured by the ^{3}H-TdR incorporation assay. As shown in Figure 5, esculetin alone exhibited little, if any, mitogenic activity on the murine splenic lymphocytes. Interestingly, esculetin significantly increased the Con A- or LPS-induced proliferation of splenic lymphocytes at 48 h of incubation.

Effect of esculetin on the induction of murine LAK cell activity

The ability of esculetin to induce LAK activity on murine splenic lymphocytes was assessed in the presence or absence of recombinant IL-2. Figure 6 shows that esculetin administered *in vivo* was able to induce the LAK activity of splenic lymphocytes and its effect was comparable to that induced by IL-2 *in vitro*. In particular, when the mice were

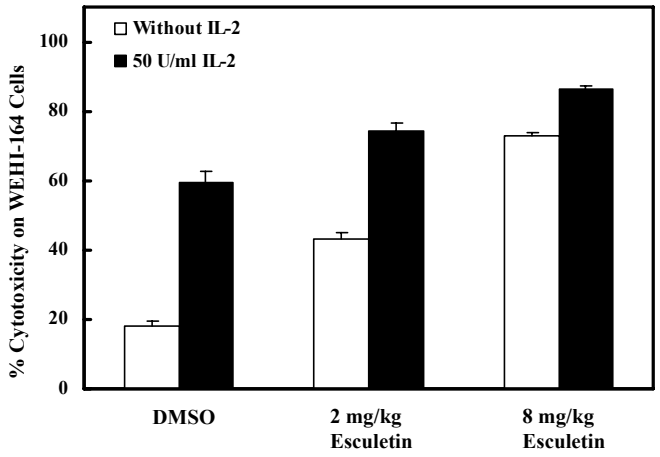


Figure 6. Effect of *in vivo* esculetin treatment on the induction of LAK activity in murine spleen cells. BALB/c mice in groups of five were either injected *i.p.* with solvent control or esculetin (2 mg/kg and 8 mg/kg). Three days later, the spleen cells were harvested and incubated with or without 50 U/ml recombinant IL-2 for 3 days at 37°C at a cell density of 2.5×10^6 cells/ml. The LAK activity was assayed by measuring the ability of the cultured spleen cells to kill the LAK-sensitive target WEHI-164 cells using the neutral red assay. The results were expressed as the % cytotoxicity on WEHI-164 cells and the data shown represented the mean ± SE of quadruplicate cultures.

treated with 8 mg/kg esculetin, the induced LAK activity by esculetin alone was greater than that induced by 50 U/ml of IL-2 in the control group as reflected by a higher cytotoxicity on the LAK-sensitive target WEHI-164 cells.

Discussion

It has been reported that esculetin, the 6,7-dihydroxy derivative of coumarin, possess cancer chemopreventive and anti-tumor activities *in vivo* (15, 16). In this paper, attempts had been made to study the modulatory effect of esculetin on the effector cells of the murine immune system, which might be one of the possible modes of action for this coumarin to exert its anti-tumor effect *in vivo*. It is known that macrophages play a key role in both innate and adaptive immunity (30). They can act as modifiers of the immune response and as non-specific effector cells capable of destroying sensitized and non-sensitized target tissues. They can also recognize tumor cells and react indiscriminately against tumors with different immunogenicities. Therefore, drugs that can enhance the activity of macrophages would be an immunomodulator with potential to fight against tumors.

Our results showed that esculetin did not exert appreciable cytotoxic effect on macrophages *in vitro* over a wide range of concentrations. Similarly, Gallicchio et al. (31) also showed that coumarin was nontoxic for human peripheral blood mononuclear cells at concentrations up to 100 μg/ml. Interestingly, esculetin was found to enhance the thioglycollate-

induced macrophage migration *in vivo*. Our results are in agreement with an earlier study showing that coumarin could significantly increase the total macrophage numbers as well as the percentage of stimulation on the skin side of the coverslip implanted subcutaneously onto rats (32). Another report also demonstrated that the number of macrophages was greatly increased in the injection site, especially with the administration of coumarin (33). The mechanism for this enhancement of macrophage migration remains obscure. One possibility might be due to the increased release of certain cytokines which are chemotactic for macrophages. However, whether esculetin can stimulate chemokine production or release awaits further investigation.

In addition to its stimulatory effect on macrophage migration *in vivo*, esculetin was found to increase the endocytic ability of LPS-activated macrophages to internalize FITC-conjugated albumin. Similarly, an earlier study had demonstrated that 20 μg/ml coumarin had a stimulating effect on macrophages to phagocytose latex particles (21). Another report also showed that 7-hydroxycoumarin, at concentrations of 2 and 20 μg/ml, caused a 124% and 84% increases in phagocytosis of human peripheral blood granulocytes and murine peritoneal macrophages respectively (23).

Apart from phagocytosis, nitric oxide production by macrophages is another parameter indicating macrophage activation. NO has been recognized as a major effector molecule involved in the destruction of tumor cells by activated macrophages (34). In the present study, esculetin alone was found to be unable to induce NO release from macrophages, but in combination with 20 ng/ml LPS, it could enhance the LPS-induced NO production in a dose-dependent manner. A recent report also demonstrated that 7-hydroxy-5,6-dimethoxycoumarin and 6,8-dihydroxy-5,7-dimethoxycoumarin are two of the most potent NO-inducers among a series of samples tested (35). The ability of esculetin to enhance the LPS-induced NO production in macrophages was further confirmed by examining the expression level of the *iNOS* gene. NO production in macrophages is related to the level of iNOS protein, which is regulated primarily at the level of gene transcription (29). The gene does not express in normal situation or the expression level is too low to be detected, while upon stimulation with macrophage activators such as LPS, its expression would be induced, so called “inducible” NOS. Our data showed that the *iNOS* gene was expressed only when macrophages were treated with LPS, and esculetin significantly enhanced the LPS-induced *iNOS* gene expression. Similarly, a report showed that oleanolic acid, a pentacyclic triterpene acid present in many medicinal plants, elicited a dose-dependent increase in NO production accompanied by an increase in iNOS mRNA expression (36).

The mechanism by which esculetin can enhance LPS-induced NO production in macrophages remains obscure. It has been reported that the induction of iNOS by LPS occurs through the activation of a toll-like receptor 4 (TLR4)-dependent signaling cascade (37). In response to stimuli, LPS can bind to membrane-localized toll-like receptors on macrophages, leading to the induction of specific signal transduction pathways, and release large amounts of NO into the general circulation to exhibit systemic effects (38, 39). It has been shown that CD14 and toll-like receptors are two membrane proteins that contribute to the regulation of NO synthesis in macrophages in response to LPS stimulation (38, 40), therefore the binding efficiency of LPS to CD14 and toll-like receptors plays a critical role in determining the induction strength with regard to iNOS. In our present study, esculetin could enhance the LPS-induced NO production, one of the speculations is that esculetin might be able to enhance the binding of LPS to the receptors so as to achieve the synergistic effect on NO production. Another speculation is that esculetin might able to increase the number of receptors for LPS binding, thus enhancing NO production. The finding that coumarins can enhance the effect of LPS for the activation of macrophages is not surprising since an earlier report also demonstrated that at 10 pg/ml LPS, which was ineffective itself, was able to induce significant amounts of interleukin-1 (IL-1) production in the presence of coumarin or 7-hydroxycoumarin (41). It was speculated that the synergism between LPS and coumarins could be used to reduce the toxic side effects of LPS in activating macrophages for tumor destruction (1).

It was reported that esculetin is a lipoxygenase inhibitor (42) and it can suppress the production of reactive oxygen species, including NO production. However, LPS, which is known to induce NO production, would also down-regulate the iNOS expression in swine heart *in vivo* (43). So the action of drug may depend on the types of tissues and cells where it acts on. The apparently contradictory effects might also reflect a differential immunomodulatory efficacy of coumarins, on one hand, reducing a potential harmful effect (respiratory burst) and on the other hand, increasing a mechanism which most probably resulting in the elimination of invading pathogens or potentially noxious cells in the case of malignant transformation (1). Actually, from the results of endocytosis and NO production, esculetin alone would have no significant effect on the macrophage activation, and an enhancing effect on macrophage activation was only observed in the presence of LPS. As LPS is a component of the outer membrane of Gram-negative bacteria and has been identified as one of the critical factors involved in the pathogenesis of sepsis (44), it represents a certain kind of pathogen invasion, therefore it is possible to say that esculetin would not affect the macrophage function in normal situation, but it could enhance the activation of macrophages when they encounter foreign invaders.

Besides macrophages, lymphocytes also play an indispensable role in our immune defense against tumors, especially as cytotoxic cells such as cytotoxic T lymphocytes, natural killer cells and LAK cells (45). In the present study, *in vivo* administration of esculetin into mice was shown to increase the mitogenesis of splenic lymphocytes towards Con A and LPS stimulations, suggesting that esculetin could increase the number of immune cells when the host encounters foreign invaders. Apart from the lympho-proliferative response of lymphocytes, LAK cell activity was shown to be induced by esculetin, and the effect was

comparable to that induced by 50 U/ml of IL-2. LAK cells have been shown to kill tumor cells *in vitro* and mediate tumor regression upon adoptive transfer into tumor-bearing mice or cancer patients (46, 47).

Collectively, our findings demonstrated that esculetin could exert immunomodulatory effects on macrophages and lymphocytes, both *in vitro* and *in vivo*. It has been speculated that by modulating the effector cells of the immune system, coumarins might result in anti-tumor responses *in vivo* (1). For examples, the macrophages can help to recognize tumor cells and present them to cytotoxic cells, and macrophages themselves can also produce NO which can promote apoptosis or sensitize tumor cells to TRAIL-mediated apoptosis (48), together with other death receptor systems such as Fas/FasL, produced by cytotoxic T cells or LAK cells, apoptosis of tumor cells can be triggered. Then macrophages would internalize and degrade the apoptotic bodies of tumor cells by phagocytosis, and result in tumor suppression.

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