Regulation of apoptosis related factors in intrinsic signaling pathway by myricetin in vitro

（ミリセチンによる内因性シグナル伝達経路のアポトーシス関連因子の試験管内での調節）

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Abstract

【Introduction】

Over the past decades, many researches are focusing on chemotherapy and radiotherapy on cancer. However, despite efforts of many researches, the severe side effects of currently available drugs can not be ignored. As a result, numerous researchers attempt to find, safe and healthy auxiliary compound to satisfy the synergistic effect with some cancer therapies such as chemotherapy, radiotherapy, etc. In the past ten years, dietary supplement manufacturers have shown strong interest in a group of compounds named polyphenols. The main reasons why polyphenols have been paid such attention are attributed to their antioxidant properties. Polyphenols, as secondary metabolites of plants, are utilized for protection against ultraviolet radiation or aggression by pathogens. Naturally, polyphenols are contained in various fruits and vegetables, especially when during the ripeness of plants. For instance, Claudine et al., (2004) pointed out that 100 g fresh weight of fruits such as grapes, apple, pear, cherries and berries contain up to 200-300 mg of polyphenols, while a glass of wine or a cup of tea contains around 100 mg of polyphenols. Over 8000 polyphenolic compounds have been identified in different kinds of plant species. According to the function of phenol rings, the distinction of polyphenols can be classified as phenolic acids, flavonoids, stilbenes, and lignans. Phenolic acids can be divided into two classes including benzoic acid and derivatives of cinnamic acid. Gallic acid, as a kind of hydroxybenzoic acid, which is contained in tea leaves up to 4.5 g/kg of fresh weight tea. Although the contents of hydroxybenzoic acids are numerous in food, the hydroxycinnamic acids are relatively more common than hydroxybenzoic acids. Flavonoids in fruits and vegetables especially quercetin, kaempferol and myricetin can be widely found even though their concentrations are as low as about 15-30 mg/kg of fresh weight. Onions is well known as the richest sources for quercetin, which contains up to 1.2g/kg fresh weight of flavonols. In opposite, the flavones are not as common substance as compared with flavonols. Stilbenes are another type of polyphenol and one of the most well studied stilbene is resveratrol. Moreover, linseed, as a representative of lignans, is the richest dietary source.

Antioxidant Effect

Polyphenols commonly exhibited its potent antioxidant property in various aspects such as cardio-protective effect, neuro-protective, anti-diabetes, anti-virus, anti-bacterial and anti-inflammatory.

Cardio-protection

Many researchers have demonstrated that consumption of polyphenols improve heart diseases. Renaud et al has already reported that wine can decrease incidence of coronary heart disease. Another report by Dubick et al showed that the polyphenols in wine and tea played as modulators of atherosclerosis and ischemic heart disease in humans. The review written by Nardini et al indicated the roles of dietary polyphenols in platelet aggregation. In detail, quercetin, an abundant polyphenol of onion was utilized in patients with ischemic heart disease (IHD) having metabolic syndrome. As a result, quercetin did not only show antiischemic and, antiarrhythmic effects, but also exhibited regulating effects on vegetative homeostassin, oxidant disturbances in patients with IHD. García-Lafuente et al reported that flavonoids, which serve as anti-inflammatory agents, can remit the symptoms of coronary heart disease by inhibiting the expression of metalloproteinase 1 (MMP1). Maeda K et al found that catechins could inhibit invasion and proliferation of the smooth muscle cells in the arterial wall that would be a mechanism, which may contribute to slow down the formation of the atheromatous lesion. Moreover, IHD, which is a leading cause of cardiac dysfunction and subsequent morbidity in the world, is characterized by impaired blood flow to a region of the heart leading to cardiac cell death. The most common manifestation of IHD which can lead to cardiac cell death is heart attack. Past studies have reported that resveratrol exhibited cardioprotection by protecting deleterious effects on the heart in the management in IHD. Raj et al have also discussed the efficacy of resveratrol on cadioprotection. In addition, many studies have been reported that catechin can improve the heart failure. For example, Zhang et al have investigated that the therapeutic effect of (-)-epigallocatechin-3-gallate (EGCG) on heart failure by inhibiting transfer membrane of GRK2 and reducing desensitization of β1-AR. Dong et al demonstrated that EPI (exocrine pancreatic insufficiency) suppressed AngII-induced cardiac hypertrophy by activating the SP1/SIRT1 signaling pathway. Oyama et al also showed green tea catechin attenuated the progression of heart failure induced by the heart/ muscle-specific deletion of MnSOD in mice. Moreover, Peters et al

also implied that the consumption of polyphenol rich diet has been associated to a lower risk of myocardial infarction in both case-control and cohort studies.

Anti-diabetes

Diabetes can be divided into two main categories: type-1 and type-2. Rizvi et al have shown that several physiological parameters of the body get altered in the diabetic conditions. Long-term effect of diabetes can lead to some progressive development of specific complements such as nephropathy and retinopathy. Previous studies have already shown anti-diabetic potential of polyphenols. Rizvi et al investigated the protective role of tea catechins against oxidation-induced damage of type-2 diabetic erythrocytes. Sahebkar et al reported the effect of curcuminoids plus piperine-modulated adipokines in type 2 diabetes mellitus. The results showed curcumin supplementation increased adiponectin, while the leptin levels were decreased and reflected a decrease in the inflammatory TNF-α level. Zheng et al reported that the polyphenol rich extract from Phellinus igniarius possessed potential anti-diabetic effects including improving glucose tolerance, reducing hyperglycemia, and normalizing insulin levels by the activation of GLUT4 translocation via the modulation of the AMPK pathway. Another polyphenol-rich naturally occurring compound named ethyl acetate fraction (EAF), which is isolated from Molineria latifolia rhizome, was utilized as dietary interventions for type 2 diabetes mellitus and its underlying molecular mechanisms in vivo. It was suggested that EAF exhibited its effect by modulating insulin signaling, potentially via IRS1/AKT activation. The pharmacological attributes of EAF may implicate its potential therapeutic applications for diabetes management. Some researchers also tried to use resveratrol to decrease insulin secretion and delay the onset of insulin resistance. Perhaps the mechanism depends on the inhibition of K + ATP and K + V channel in β-cells. It is clearly known that quercetin a dominant polyphenol of onion, has already exhibited its protective effects on the alterations in diabetic patients by significantly protecting the lipid peroxidation and inhibiting antioxidant system in diabetics. Another polyphenol that is abundant in maize bran, is regarded as a potent anti-diabetic agent by lowering blood glucose followed by significantly increased plasma insulin and a negative correlation between blood glucose and plasma insulin. Many other studies also showed that polyphenols exhibit potential in relievinge the symptoms of diabetes by acting in different levels including inhibiting intestinal glycosidases and glucose transporter, decreasing of S-Glut-1 mediated intestinal transport of glucose, delaying the

transfer of glucose from stomach to the small intestine and modulating SIRT1 by improving whole-body glucose homeostasis and insulin sensitivity in diabetic rats.

Anti-inflammatory

Inflammatory, a process by which white blood cells attempt to combat injury or foreign organisms such as virus, bacteria or other pathogens, is a kind of immune defense mechanism. It is always accompanied by some symptoms such as redness, swelling, pain, stiffness and loss of joint function. Large amounts of anti-inflammatory drugs have been already widely used such as aspirin, diclofenac, ibuprofen, acetaminophen, and paracetamol. However, unexpected side effects commonly occurred after using medicines. Thus, some researchers tried to develop safer compounds and mechanism-based approaches for the management of diseases. Polyphenols have been proved in recent several decades as anti-inflammatory agents. Yoon et al focused on some pivotal molecular targets of polyphenols that directly affect the inflammation process. Joseph et al made a summary providing a comprehensive overview of human clinical trials which investigated the acute and chronic effects of polyphenols from commonly consumed fruits or their derived products on inflammation. Additionally, some researchers utilized a polyphenols-rich extract from tea (Camellia sinensis) flowers to treat inflammatory in acute and chronic mice models. Santos et al used a natural polyphenol, chlorogenic acid to evaluate the anti-inflammatory, analgesic and antipyretic activities. Some researchers summarized the effects of flavonoids and other polyphenols on inflammation. Recently, some novel studies showed flavonoids exhibit its anti-inflammatory potential in neurodegenerative disorders. They found pure flavonoids or enriched-extracts function as anti-inflammatory agents by reducing the expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β and COX-2), down-regulating inflammatory markers and preventing neural damage. This effect is mainly related to the regulation of microglial cells, mediated by MAPKs and NF-κB signalling pathways on the basis of their in vitro and in vivo experimental results. It was implied the role of inflammation in neurodegenerative diseases, and the potential therapeutic effects of flavonoids as a promising approach to develop innovative neuroprotective strategy.

Prooxidant Effect

Perhaps most of researchers paid attention about anti-cancer effects of polyphenols because their pro-oxidant property. And the pro-oxidant effect plays the most important role in the

mechanism of cancer protective process by inducing reactive oxidative stress. Various studies have reported that polyphenols exhibit its potential for cancer therapy reagents as major or auxiliary therapeutic drug with chemotherapy or radiotherapy. The anti-cancer effect can be realized by reducing the numbers and growth of tumors. Numerous polyphenols showed their protective effect in some models even though their mechanisms of action were found to be different. García-Lafuente et al have identified for chemoprevention effects of polyphenols by mechanisms of action including estrogenic/anti-estrogenic activity, anti-proliferation, formation of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity and changes in cellular signaling. Past studies have demonstrated the combined effects of polyphenols with other anticancer drugs or therapies. For example, Lewandowska et al reported synergistic interactions between anticancer chemotherapeutics and phenolic compounds. Additionally, Fantini et al reported that the anticancer effects of combinations of polyphenols or polyphenols and anticancer drugs, with a focus on their ability to modulate multiple signaling transduction pathways involved in cancer. A review focusing on the dietary polyphenols and prevention of diseases showed that polyphenols modulate the expression of cytochrome P450 enzymes involved in the activation to carcinogens so as to influence the metabolism of pro-carcinogens. Potentially toxic quinones which are substrates of these enzymes can be formed by polyphenols. In addition, these enzymes can be activated by the intake of polyphenols for their detoxication to induce a general boosting of our defenses against toxic xenobiotics. The cancer preventive activity was shown by administration of polyphenol to inhibit the conversion of high grade prostate intraepithelial neoplasia (PIN) to men having high-grade PIN. Another polyphenol named as myricetin also exhibited anticancer on human anaplastic thyroid cancer via mitochondria dysfunction. Moreover, myricetin increased the cytotoxicity of paclitaxel due to the significant down-regulation of MDR-1 in these cells when it was used for pre-incubation of ovarian cancer cells in lower dose manner. Yang et al also proved that myricetin suppresses invasion and promotes cell death in human placental choriocarcinoma cells through induction of oxidative stress. In addition, quercetin does not only possess anticancer property against benzopyrene induced lung carcinogenesis in mice, but also its free radical scavenging activity. These studies provided a potent proof for supporting the use of dietary polyphenols in human cancer chemoprevention, in a combinational approach with either chemotherapeutic drugs or cytotoxic

factors for efficient treatment of drug refractory tumor cells. Polyphenols often show anti-carcinogenetic effects by the induction of apoptosis mediated by signaling pathways especially mitochondrial or intrinsic signaling pathway to protect all stages of development of cancer. Mitochondria, which plays a critical role in apoptosis, is closely associated with some death signals and trigger apoptosis by releasing death factors into cytosol, such as cytochrome c. Thus, in the following study, one kind of commonly natural occurring polyphenol named myricetin is utilized for clarifying its prooxidant property mediated by intrinsic signaling pathway.

【Methods】

PC12 cells were treated with myricetin in two concentration levels comprising 0.1 and 1 μM under serum-free condition. MCF-7 cells were treated with three concentration levels including 10, 20 and 40 μM. Morphological changes were observed using trypan blue assay. DNA fragmentation was determined by DNA ladder assay after treatment of myricetin in PC12 cells under serum deprivation condition. IC50 by MTT assay and GSH levels were determined for analyzing cytotoxicity and oxidative stress after treatment of myricetin in MCF-7 cells. The expression of cytochrome c, p53, Bax, Bcl-2, caspase-3 and 9 were determined by western blot analysis in the experiment of PC12 cell. In the experiment of MCF-7 cells, the expression of p53, NF-kB, Bax, Bcl-2, Bcl-xL, Apaf-1 and caspase-3 were determined by western blot analysis.

【Discussion】

Polyphenols have been widely studied for their antioxidant properties, especially preventing damage from reactive oxygen species (ROS) that formed as by-products of mitochondrial respiration or by certain oxidases or preventing generation of these species including hydrogen peroxide (H_2O_2), superoxide anion (O_2•−), and hydroxyl radical (OH•). They are active in many cellular events, including as second messengers in the activation of several signaling pathways leading to the activation of transcription factors, mitogenesis, gene expression, and the trigger of apoptosis. Polyphenols show antioxidant property in various aspects such as anti-histamine, anti-inflammatory, antibacterial, and antiviral activities. However, it is more important to point out that polyphenols also exhibit its potent prooxidant property in lots of in vitro assays, anticancer effect in particular. In order to reduce the risk of chronic diseases have pointed much research to discern the lifestyle choices that potentially stimulate developing chronic pathologies, especially cancer. Epidemiological evidence has been already reported that the daily consumption of fruits

and vegetables can reduce the risk of developing malignancies, especially, the polyphenol components of phytochemicals have been identified as anticarcinogens. Additionally, glutathione (γ-glutamylcysteinylglycine; GSH) plays a critical role in maintaining intracellular redox balance and alleviating ROS-induced oxidative stress. A major function of GSH is to scavenge ROS and thereby to prevent oxidative damage. Because oxidative stress has been implicated with cancer, as well as with other chronic diseases and pathologies, including atherosclerosis, neurodegenerative diseases, and aging, much research has focused on the antioxidant properties of plant derived polyphenols. Interestingly, naturally occurring antioxidant polyphenols exhibit both pro-oxidative and antioxidative properties, depending on the factors as their metal-reducing potential, chelating behavior, pH, and solubility characteristics.

It was reported that either green tea or with red wine inhibited proliferation of rat pheochromocytoma PC12 cells through generating H_2O_2. And the addition of catalase completely abolished the antiproliferative effects of green tea, but only partially reduced that of red wine. It was suggested that the toxicity of red wine was a combination of H_2O_2 with resveratrol, the main polyphenol in red wine, which has already known its antiproliferative effects. Some researchers, using PC12 cells which derived from a pheochromocytoma of the rat adrenal medulla, showed that the level of EGCG was critical in evoking cell death by apoptosis. Low levels of EGCG (e.g., 50 μM) apparently induced mild oxidative stress, while a higher level EGCG (e.g., 400 μM) stimulated oxidative stress, as indicated by a persistent elevated intracellular level of ROS, induce disruption of the intracellular GSH level and an increase in lipid peroxidation. In the present study, myricetin showed no effect on the apoptosis in PC12 cells under 10% FBS condition. But enhansive effect was exhibited on the apoptosis induced by serum deprivation in PC12 cells. It is necessary to point out that the grace doses of myricetin (0.1 and 1 μM) were selected in this study.

Previously, Hsuuw and Chan reported that the effect of EGCG with moderate (20–50 μM) and to high levels (100–400 μM) in human breast cancer MCF-7 cells. As a result, apoptosis was induced when MCF-7 exposured to moderate level of EGCG, while necrosis was induced when exposured to high level of EGCG. After MCF-7 cells being exposured to moderate levels of EGCG, cell viability was decreased, and apoptosis was induced. It is found a closed relation with increased oxidative stress, as indicated by intracellular generation of ROS, a loss of mitochondrial membrane potential, activation of caspase-3, caspase-9, and c-Junterminal kinase (JNK), and an

increased expression level of Bax protein and a decreased level of Bcl-2 protein, finally shifting the Bax versus Bcl-2 ratio to trigger apoptosis. However, little levels of ROS and of apoptotic cells and only minor effects on caspase activation, Bax versus Bcl-2 ratio, and on mitochondrial membrane potential were showed. Cell death correlated with leakage of LDH, a sign of necrosis. Lowered ATP levels were observed at the high levels of EGCG. It was suggested that the switch from apoptotic death to necrosis was controlled by the intracellular level of ATP, with high ATP levels favoring apoptosis and decreased levels favoring necrosis. Herein, myricetin showed an enhansive effect on the apoptosis induced by serum deprivation but not normal serum condition in PC12 cells, and moreover a potent toxicity on breast cancer MCF-7 cell lines which was also noted by the induction of apoptosis. It is possible to point out that the sensitivity of two cell line is different. However, both of the apoptosis mechanisms are consistent. For instant, in the MCF-7 experiment, cell viability results showed cell death induction in a dose dependent manner. Western blot results implied the trigger of apoptosis, which was induced by myricetin in a dose dependent manner. Our past study has already reported myricetin could enhance apoptosis induced by serum deprivation in PC12 cells mediated by mitochondrial signaling pathway. Serum starvation, as external stimuli, triggered the activation of p53. And p53-induced apoptosis in the way of regulating transcription of pro-apoptotic Bcl-2 family members like Bax which has been already investigated by Miyashita and Reed previously. Moreover, p53 activation evokes a series of complicated and closed link to execute apoptosis by the release of cytochrome c. In fact, the release of cytochrome c, which is induced by the transcriptional activation of Bcl-2 family members like pro-apoptotic Bax and anti-apoptotic Bcl-2, triggers the downstream caspase cascade reaction and apoptogenic protein expression. Generally, the released cytosolic cytochrome c which binds with the adaptor molecular Apaf-1, forms a complex with oligomerizated caspase 9 in the presence of ATP to activate caspase-3 and sequentially cleave cellular death substrates. Herein, a consistent mechanism of apoptotic signaling pathway was demonstrated. Myricetin, serve as toxic stimuli for MCF-7 cells, induced gradual cell death in a dose dependent manner which was shown from MTT assay and cell viability results. In this process, myricetin stimulated more ROS generation, which can be reflected by the lowering of GSH levels. The changes of GSH levels imply the imbalance of redox in intracellular environment. The overload of ROS triggered the activation of p53. Previous study already investigated the suppression of p53 activation could

contribute in the role of NF-κB in tumorigenesis and other diseases. In present study, the activation of p53 attenuated the role of NF-kB contrarily and induced the transcriptional activation of Bax versus Bcl-2 dimers. Additionally, the protein expression of anti-apoptotic Bcl-x also showed a decreasing trend. Moreover, the downstream caspase cascade reaction was stimulated like caspase-3 protein expression. Indeed, myricetin regulated apoptosis related factors such as NF-κB, p53, Bax, Bcl-2, Bcl-xL, cytochrome c, Apaf-1, caspase-3 and 9 in the intrinsic signaling pathway in vitro.

The locations of hydroxyl groups play important to the biological activities of flavonoid. Previous study reported hydroxyl groups at C3', C4' and C5' are important for the cytotoxic effect of myricetin on HL-60 human promyeloleukemic cells and THP-1 mature monocytic cells. If OH groups which located at C3', C4' and C5' were deleted such as galangin or replaced by OCH_3, both of their IC50 were higher than myricetin. It demonstrated that the location of hydroxyl groups at C3', C4' and C5' exhibited higher level of toxicity. It was pointed out that the hydroxylation at C4' or C6 plays an essential role for apoptosis-inducing activity of flavanone through activation of caspase-3 cascade and production of reactive oxygen spiecies. Large numbers of external stimuli induced apoptosis via ROS production although there was no ROS production determined by several ROS assay systems in Ching's study. However, ROS, function as mitogens, can also induce proliferation and protect cells from apoptosis induced by oxidative spiecies. These data indicates ROS exhibits double-sided fuction. In the present study, the redox balance was disrupted by myricetin according to GSH peroxidase into GSSG because of ROS production. On the contrary, most of flavonoids with multiple OH substitutions like quercetin have exhibited mutagenic in vitro through prooxidant rather than antioxidant action.

【Conclusion】

The present research exhibited the potent inhibitory effect of myricetin on the growth of PC12 cells and MCF-7 cells through apoptotic signaling pathways. Herein, the activation of p53 plays a critical role for triggering a series of molecular biological changes including disruption on the balance of intracellular redox environment and some apoptosis related proteins such as p53, NF-κB, Bax, Bcl-2, cytochrome c, caspases-9 and 3. The anticancer mechanism of most anticancer drug or polyphenols depends on intrinsic signaling pathway as mentioned in the past chapter. Mitochondria played a central role in the intrinsic signaling pathway. The mitochondrial

membrane potential causes increase of mitochondrial voltage-dependent anion channel (VDAC), loss of mitochondrial outer membrane potential and damage on PTP and finally opening of the permeability transition pore. This is an irreversible step toward apoptosis, which has been reported. Intermembranous mitochondrial protein cytochrome c was released from mitochondria into cytosol. Cytosolic cytochrome c, binded with Apaf-1, formed a complex with procaspase-9 to activate caspase-3. The disruption of mitochondrial membrane permeability was triggered by the transcriptional activation of Bax versus Bcl-2 dimers, which directly induced by p53 activation. DNA fragmentation of PC12 cells was determined implicating the occurrence of apoptosis. Herein, myricetin did not induce apoptosis under normal condition (10% FBS), but enhanced apoptosis induced by serum deprivation in PC12 cells. Furthermore, myricetin directly induced apoptosis in MCF-7 cell lines. It is possible that the sensitivity of PC12 cells is lower than MCF-7 cells.

It is well known that myricetin exhibited its potent anti-oxidant property to protect from ageing. However, more potential prooxidant function should be paid attention as the data shown in the present study. Further study is needed for clarifying the further application of myricetin in vivo and in clinical studies. It can be predicted that the absorbed concentration of myricetin will be different with intake concentration due to its digestion and metabolism. Generally, this study provided more evidence for clarifying the prooxidant effect of myricetin through intrinsic signaling pathway.