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Involvement of Caspase-8/ Caspase-9/ Cytochrome-c in Caspase-3-dependent Apoptosis Induced by Kaempferol and Quercetin in Oral, Breast and Colon Cancer Cells

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Abstract

This study aims to test whether caspase-8, caspase-9 and cytochrome-c are involved in caspase-3-dependent apoptosis induced by kaempferol and quercetin in oral, breast, and colon cancer cells. This paper reports the devised experiments based on cell proliferation assays, flow cytometry, and Western Blot analysis. It also discusses 12 possible results of the experiments as well as their implications on the study of apoptotic mechanism of kaempferol and quercetin.

Keywords

Kaempferol; Quercetin; Caspase-3; Caspase-8; Caspase-9.

1. INTRODUCTION

The Oriental medicine has for a long time been used by East Asians as a treatment to various discomforts, including headaches, fatigue and hypertension. Among the different herbs used in Oriental medicine, Gingko biloba is widely studied because of its capability of treating neural diseases – such as ischemic stroke[1]and amyloid aggregation[2] – and repairing damaged vessels[3], with the mechanisms being deeply investigated.

As one may expect, in the treatment of cancer, a disease resulted from uncontrollable division of body cells, Oriental medicine is also being studied as one potential treatment to prevent the fatal disease from deteriorating. Recent research has shown that EGb761, a type of extract of Ginkgo biloba, is able to inhibit the growth and proliferation of cancer cells, including colon, gastric and breast cancer [4-6]. As suggested by the research, the antiproliferation effect on these cancer cells achieved by EGb761 is due to cell apoptosis, in which a certain type of protein called caspase-3 is involved.

Caspase-3, a member of the caspase family, is an important enzyme in mammals which is relative to processes such as cell apoptosis and inflammation. Cell apoptosis is a biological process that involves the removal of unwanted cells, which is crucial to the development of organisms. During caspase-3-dependent cell apoptosis, caspase-3 can be activated with or without other caspases such as caspas-8 and caspase-9, which is triggered by death stimuli resulted from the binding of death ligands or the release of cytochrome-c. When activated, caspase-3 will have the ability to cause DNA fragmentation and other changes that eventually leads to cell apoptosis. [7, 8]

In a recent study, the ability of EGb761 to inhibit proliferation and induce caspase-3-dependent apoptosis in oral cavity cells have been proved.[9]However, it is still important to have a further insight into the components of EGb761 and to determine which component of it is responsible for caspase-3-dependent cell apoptosis. If apoptosis is indeed caused by certain components of EGb761, the dose can be more efficient and the side effect can be minimized. In

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the study carried out by Kang et al., the researchers used three types of oral cavity cancer cell lines as well as their culture, and measured their proliferation, apoptosis and caspase-3 activity. The result of cell proliferation measurement suggested that, among all of the components of EGb761, kaempferol and quercetin are responsible for the anti-proliferation effect. In addition, flow cytometry and Western blot analysis results both confirmed that the anti-proliferation effect was due to cell apoptosis. What's more, the result of the study clearly reveals that the cell apoptosis induced by kaempferol and quercetin involves caspase-3. [10]Other studies also show that treatment containing kaempferol and quercetin is able to induce apoptosis in breast and colon cancer cells, involving caspase-3. [11]

Since caspase-8, caspase-9 and cytochrome-c are also important factors in caspase-3 dependent cell apoptosis, it is reasonable to hypothesize that they also contribute in apoptosis induced by kaempferol and quercetin. This project is a further development of the studies mentioned above. It aims to study the role caspase-8, caspase-9 and cytochrome-c play in caspase-3-dependent cell apoptosis in oral, breast and colon cancer cells.

2. METHODOLOGY

The purpose of the study is to test whether caspase-8, caspase-9 or cytochrome-c is involved in caspase-3-dependent apoptosis in different cancer cells induced by kaempferol and quercetin. The cells will be divided into different groups, each containing cultures of oral, breast and colon cancer cells, as well as different lines of them. Control groups without treatment will be set. In addition, a preliminary experiment with cell proliferation assays will be conducted, in order to determine the most suitable concentration of kaempferol and quercetin solutions. The concentration gradient will be set as 5nM, 10uM, 20uM, 40uM, 80uM and 160uM. Assume the final concentration is XuM. In order to reduce the errors, each trial will be performed three times. Statistic methods will also be used to confirm significance.

Two objectives should be achieved in the study.

2.1. Objective 1

Test whether caspase-8 and caspase-9 are involved in cell apoptosis induced by kaempferol and quercetin. Four groups will be treated with XuM of kaempferol and XuM of quercetin, with z-IETD-fmk and z-LEHD-fmk respectively. The other two groups will be treated with equal amount of kaempferol or quercetin, but without z-IETD-fmk or z-LEHD-fmk. Another two groups will be treated with equal amount of kaempferol or quercetin, with both z-IETD-fmk and z-LEHD-fmk in each group. Apoptosis in these cells will be examined through flow cytometry. In addition, PARP cleavage analysis will also be performed, which will involve Western Blot analysis.

2.2. Objective 2

Test whether cytochrome-c is involved in cell apoptosis induced by kaempferol and quercetin. Each group will first be treated with XuM of kaempferol and XuM of quercetin respectively. Then, the apoptotic cells will be separated using flow cytometry. These cells will then be lysed and centrifuged in order to extract their cytosol. After that, the WB analysis for cytochrome-c will be performed on the extracted cytosol, and the presence of cytochrome-c will be examined.

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Table 1. Group division of the experiment.

	Group I XuM kaempferol	Group II XuM quercetin	Group III No treatment
+ z-IETD-fmk	Group A1 for flow cytometry Group A2 for WB	Group A3 for flow cytometry Group A4 for WB	Group A5 for flow cytometry Group A6 for WB
+ z-LEHD-fmk	Group B1 for flow cytometry Group B2 for WB	Group B3 for flow cytometry Group B4 for WB	Group B5 for flow cytometry Group B6 for WB
- z-IETD-fmk / z-LEHD-fmk	Group C1 for flow cytometry Group C2 for WB	Group C3 for flow cytometry Group C4 for WB	Group C5 for flow cytometry Group C6 for WB
+ z-IETD-fmk & z-LEHD-fmk	Group D1 for flow cytometry Group D2 for WB	Group D3 for flow cytometry Group D4 for WB	Group D5 for flow cytometry Group D6 for WB
Cyt c	Group E1	Group E2	Group E3

3. RESULTS AND DISCUSSION

To begin with, cell apoptosis can be either extrinsic and intrinsic. The activation of caspase-8 is vital to the extrinsic cell apoptosis, in which binding of death receptor ligands is also involved, because the activated caspase-8 is able to activate effector caspases like caspase-3 and thus induce cell apoptosis. Meanwhile, extrinsic caspase-3-dependent cell apoptosis can also be independent of caspase-8. In the intrinsic pathway, effector caspases are activated by the release of proteins such as cytochrome-c from mitochondria, involving caspase-9. [7, 8]As we can see, in caspase-3-dependent cell apoptosis, caspase-8, caspase-9, and cytochrome-c have significant influence.

Earlier studies have shown that treatment containing kaempferol and quercetin is able to induce caspase-3-dependent apoptosis in oral cavity cancer, breast cancer and colon cancer cells. [10, 11]Another study has shown that, under the treatment of kaempferol, quercetin, and other components, both extrinsic and intrinsic pathways are activated, resulting in the apoptosis of colon cancer cells. [12]However, the exact mechanism behind the caspase-3-dependent apoptosis induced only by kaempferol and quercetin remains unclear, which inspired this study. The result of the project will further deepen the understanding of the antitumous effect of kaempferol and quercetin, which is meaningful to the further investigation of potential therapies of cancer.

There are many possible results for this research. Note that, since Group III is the control group, if the experiments are properly conducted, no apoptosis will be detected in groups A5, B5, and C5, only one 116-kDa band will be shown in the WB of groups A6, B6, and C6, no band will be shown in Group E3.

Except for cases of experimental errors, the possible conclusions and corresponding results for Group I are listed below.

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3.1. Possible Result 1

Caspase-8, caspase-9 and cytochrome-c are all involved, and two pathways are responsible for caspase-3-dependent apoptosis.

In this case, apoptosis will be detected in Groups A1, B1, and C1 but not D1. However, there will be more apoptotic cells in Groups C1 than Groups A1 and B1. A 116-kDa and a 85-kDa band will be shown in Groups A2, B2, and C2 but only one 116-kDa band will be shown in D2. However, the color of the 85-kDa band in Group C2 will be deeper than that in Groups A2 and B2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is able to activate caspase-3 by both caspase-8 (extrinsic) and caspase-9 (intrinsic) pathways, which is consistent to the study mentioned.

3.2. Possible Result 2

Caspase-8, caspase-9 and cytochrome-c are all involved, and only one pathway is responsible for caspase-3-dependent apoptosis.

In this case, apoptosis will not be detected in Groups A1, B1, and D1, but it will be detected in Group C1. A 116-kDa and a 85-kDa band will be shown in Group C2, while only one 116-kDa band will be shown in Groups A2, B2 and D2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is probably able to activate caspase-3 through a pathway "stimuli → caspase-8 → cytochrome-c → caspase-9 → caspase-3", although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.3. Possible Result 3

Caspase-8 and caspase-9 are involved, while cytochrome-c is not involved, and two pathways are responsible for caspase-3-dependent apoptosis.

In this case, apoptosis will be detected in Groups A1, B1, and C1 but not D1. However, there will be more apoptotic cells in Groups C1 than Groups A1 and B1. A 116-kDa and a 85-kDa band will be shown in Groups A2, B2, and C2, but only one 116-kDa band will be shown in D2. However, the color of the 85-kDa band in Group C2 will be deeper than that in Groups A2 and B2. No band will be shown in WB of Group E1.

Such result indicates that, kaempferol is able to activate caspase-3 by both caspse-8 (extrinsic) pathway and a "stimuli → caspase-9 → caspase-3" pathway, although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.4. Possible Result 4

Caspase-8 and caspase-9 are involved, while cytochrome-c is not involved, and only one pathway is responsible for caspase-3-dependent apoptosis.

In this case, apoptosis will not be detected in Groups A1, B1 and D1, but it will be detected in Group C1. A 116-kDa and a 85-kDa band will be shown in Group C2, while only one 116-kDa band will be shown in Groups A2, B2 and D2. No band will be shown in WB of Group E1.

Such result indicates that, kaempferol is probably able to activate caspase-3 through a pathway "stimuli → caspase-8 → caspase-9 → caspase-3", although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.5. Possible Result 5

Caspase-8 and cytochrome-c are involved, while caspase-9 is not involved, and two pathways are responsible for caspase-3-dependent apoptosis.

In this case, apoptosis will be detected in Groups A1, B1, C1, and D1. However, there will be more apoptotic cells in Groups B1 and C1 than Groups A1 and D1. A 116-kDa and a 85-kDa band will be shown in Groups A2, B2, C2, and D2. However, the color of the 85-kDa band in Group B2

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and C2 will be deeper than that in Groups A2 and D2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is able to activate caspase-3 by both caspse-8 (extrinsic) pathway and a "stimuli → cytochrome-c → caspase-3" pathway, although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.6. Possible Result 6

Caspase-8 and cytochrome-c are involved, while caspase-9 is not involved, and only one pathway is responsible for caspase-3-dependent apoptosis

In this case, apoptosis will not be detected in Group A1 and D1, but it will be detected in Groups B1 and C1. A 116-kDa and a 85-kDa band will be shown in Groups A2 and D2, while only one 116-kDa band will be shown in Groups B2 and C2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is probably able to activate caspase-3 through a pathway "stimuli → caspase-8 → cytochrome-c → caspase-3", although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.7. Possible Result 7

Caspase-8 is involved, while caspase-9 and cytochrome-c are not involved.

In this case, apoptosis will not be detected in Group A1 and D1, but it will be detected in Groups B1 and C1. A 116-kDa and a 85-kDa band will be shown in Groups B2 and C2, while only one 116-kDa band will be shown in Groups A2 and D2. No band will be shown \in WB of Group E1.

Such result indicates that, kaempferol is able to activate caspase-3 by caspase-8 (extrinsic) pathway.

3.8. Possible Result 8

Caspase-8 is not involved, while caspase-9 and cytochrome-c are involved.

In this case, apoptosis will not be detected in Groups B1 and D1, but it will be detected in Groups A1 and C1. A 116-kDa and a 85-kDa band will be shown in Groups A2 and C2, while only one 116-kDa band will be shown in Groups B2 and D2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is able to activate caspase-3 by caspase-9 (intrinsic) pathway.

3.9. Possible Result 9

Caspase-8 and cytochrome-c are not involved, while caspase-9 is involved.

In this case, apoptosis will not be detected in Group B1 and D1, but it will be detected in Groups A1 and C1. A 116-kDa and a 85-kDa band will be shown in Groups A2 and C2, while only one 116-kDa band will be shown in Groups B2 and D2. No band will be shown in WB of Group E1.

Such result indicates that, kaempferol is probably able to activate caspase-3 through a pathway "stimuli → caspase-9 → caspase-3", although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.10. Possible Result 10

Caspase-8 and caspase-9 are not involved, while cytochrome-c is involved.

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In this case, apoptosis will be detected in Groups A1, B1, C1, and D1. A 116-kDa and a 85-kDa band will be shown in Groups A2, B2, C2 and D2. A 15-kDa band will be shown in WB of Group E1.

Such result indicates that, kaempferol is probably able to activate caspase-3 through a pathway "stimuli → cytochrome-c → caspase-3", although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.11. Possible Result 11

Neither caspase-8, caspase-9 or cytochrome-c is involved.

In this case, apoptosis will be detected in Groups A1, B1, C1 and D1. A 116-kDa and a 85-kDa band will be shown in Groups A2, B2, C2, and D2. No band will be shown in WB of Group E1.

Such result suggests that kaempferol is probably able to activate caspase-3 without the help of other caspases, although no study (based on my current knowledge) have reported such apoptotic pathway yet.

3.12. Possible Result 12

The result varies between different cell types.

The result could also vary between different cell types or cell lines. In such cases, the mechanism should be determined specifically for each type of the cells with methods similar to those mentioned above.

The results in Group II (quercetin treatment) also have such possibilities mentioned above, and can be analyzed using similar approach.

4. CONCLUSION AND LIMITATIONS

The result of this study is able to provide insight into the mechanism, either consistent with previous studies [12] or not, of caspase-3-dependent cancer cell apoptosis induced by kaempferol and quercetin. The result may inspire new investigations of apoptosis mechanisms, as well as new thoughts on tumor therapies targeting caspases in oral cavity cancer, breast cancer and colon cancer cells.

However, further research aims to investigate more specific mechanisms is needed for perfection. In addition, the interactions of different pathways are not fully considered and investigated in this study, which needs further studies.

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