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Enhancement of (−)-epigallocatechin-3-gallate and theaflavin-3-3′-digallate induced apoptosis by ascorbic acid in human lung adenocarcinoma SPC-A-1 cells and esophageal carcinoma Eca-109 cells via MAPK pathways

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ABSTRACT

Tea polyphenols (−)-epigallocatechin-3-gallate (EGCG) and theaflavin-3-3′-digallate (TF_3) are two prospective compounds in cancer prevention and treatment. Ascorbic acid (Vc) is essential to a healthy diet as well as being a highly effective antioxidant. In this work, the effects of the combination of EGCG or TF_3 with Vc on the apoptosis and caspases-3/9 activities in human lung adenocarcinoma SPC-A-1 cells and esophageal carcinoma Eca-109 cells were determined. Furthermore, the role of mitogen-activated protein kinases (MAPK) pathways in the apoptosis induced by TF_3 or EGCG together with Vc were studied using three MAPK inhibitors (ERK inhibitor PD98059, JNK inhibitor SP600125 and p38 inhibitor SB203580). Our results showed that Vc could enhance the EGCG and TF_3 induced apoptosis in SPC-A-1 and Eca-109 cells, and this effect involved the activation of caspase-3 and 9. EGCG, TF_3 and Vc could activate MAPK pathways respectively, and each compound activated different MAPK subfamilies in different cells. This may explain the enhancement of EGCG and TF_3 induced apoptosis by Vc in SPC-A-1 and Eca-109 cells, and will ultimately aid the design of more effective anti-cancer treatments.

1. Introduction

Tea is one of the most popular beverages in the world. Numerous studies have shown the anti-cancer effect of tea *in vitro*, *in vivo* and in clinical trials [1,2], and this activity was mainly attributed to tea polyphenols [3–6]. (−)-Epigallocatechin-3-gallate (EGCG) and theaflavin-3-3′-digallate (TF_3) are two prospective polyphenols in tea for prevention and treatment of cancer [1–5]. EGCG is the most abundant catechin and major biologically active component in green tea. TF_3 is one of the theaflavins which are products of polymerization and oxidation of catechins in black tea. Both EGCG and TF_3 have strong reducibility since they contain multiple phenolic hydroxyl groups [7,8]. Several studies showed that EGCG and TF_3 could inhibit the initiation, promotion and progression of carcinogenesis [6,9].

Ascorbic acid (Vc) is a highly effective antioxidant due to its strong ability of donating electron. It also acts as an enzyme cofactor for the biosynthesis of many important biochemicals such as collagen. Due to these two properties, Vc helps to protect against mutagenesis and carcinogenesis [10].

The anti-cancer mechanisms of tea polyphenols and Vc are that they not only scavenge free radicals which can lead to the damage of macromolecules, but also regulate signal transduction pathways [11]. One of the important signal transduction pathways associated with cancer is the mitogen-activated protein kinases (MAPKs) pathway. MAPKs are a superfamily of proline-directed serine/threonine protein kinases [12], which includes the extracellular signal-regulated kinases (ERKs) and the stress-activated protein kinases (SAPKs). SAPKs subfamily encompasses c-Jun N-terminal kinases (JNKs) and p38. Downstream targets of MAPKs comprise mitogenic/proinflammatory enzymes and nuclear transcription factors. Thus, MAPK pathways are involved in a series of cell events, including gene expression, cell proliferation, cell differentiation, and apoptosis. Li and Tu [13] have reviewed the effects of tea polyphenols and their polymers on MAPK signaling pathways in cancer cells. The concentration of EGCG for activating p38 was 50 μM [14].

Our previous work showed that EGCG and TF_3 could induce the apoptosis of several cancer cell lines. TF_3 + Vc and EGCG + Vc had synergistic inhibitory effects on the proliferation of SPC-A-1 cells, and significantly held SPC-A-1 cells in G0/G1 phase [6]. However, the signal pathways involved in apoptosis induced by TF_3 or EGCG in combination with Vc were rarely reported. In this study, we

Abbreviations: EGCG, (−)-epigallocatechin-3-gallate; TF3, theaflavin-3-3′-digallate; Vc, ascorbic acid; MAPK, mitogen-activated protein kinases.

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investigated the enhancement of EGCG and TF_3 induced apoptosis by Vc in human lung adenocarcinoma SPC-A-1 cells and esophageal carcinoma Eca-109 cells. Furthermore, the role of MAPK pathways in the apoptosis induced by TF_3 or EGCG together with Vc were studied.

2. Materials and methods

2.1. Chemicals and reagents

TF_3 (92.4% purity) monomer was isolated and purified using our previously established method [15]. EGCG (98% purity) was purchased from Hangzhou Gosun Biotechnologies Co., Ltd. (Hangzhou, China). Vc was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Human lung adenocarcinoma SPC-A-1 cells and human esophageal carcinoma Eca-109 cells were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). MAPK inhibitors (ERK inhibitor PD98059, JNK inhibitor SP600125 and p38 inhibitor SB203580), Hoechst 33258, Caspase-3 and Caspase-9 activity assay kits were purchased from Beyotime institute of Biotechnology (Nantong, China).

2.2. Cell culture

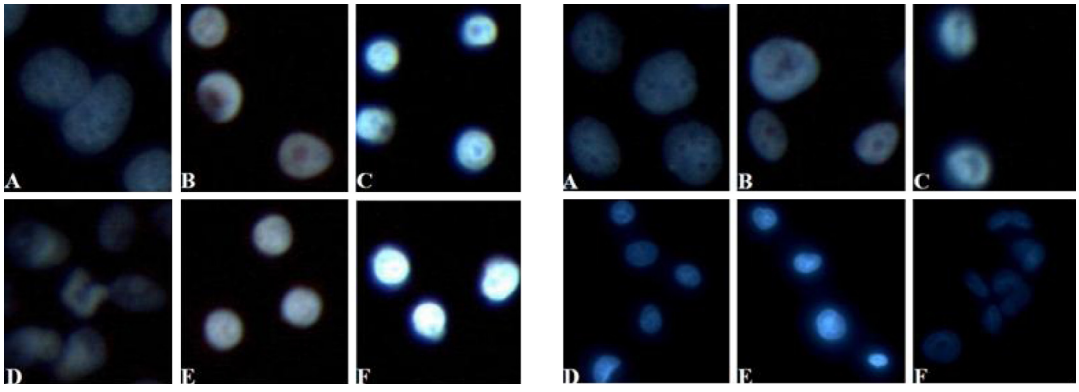
SPC-A-1 cells and Eca-109 cells were cultured in RPMI-1640 medium containing 10% FBS. The cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.3. Measurement of apoptotic morphology

Exponentially growing cells were seeded on glass coverslips placed in six-well plates. When cell confluence reached 50–80%, the cells were treated with EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc at the final concentration 50 μM of each compound for 24 h. Subsequently, cells were fixed in 4% formaldehyde for 10 min, washed with PBS twice, and then stained with Hoechst 33258 for 5 min in an incubator. Morphological changes of nuclei were assessed under a laser scanning confocal microscope (Leica TCS SP5, Germany).

2.4. Measurement of caspase-3 and caspase-9 activities

The caspase-3 and 9 activities were measured with caspase-3 and 9 activity assay kits according to the manufacturer's instructions. Cells were seeded in 96-well cell culture microplates at 1×10^4 cells/well, allowed to attach to the bottom, and then treated with EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc at the final



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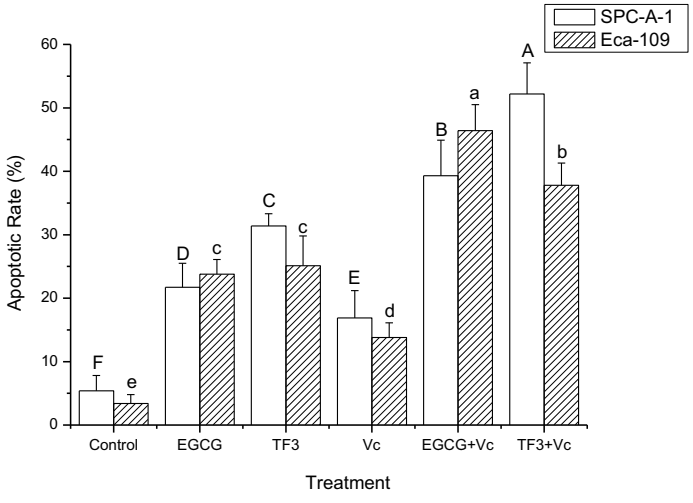

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Fig. 1. EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc induce apoptosis in SPC-A-1 and Eca-109 cells. (I) Hoechst 33258 staining of SPC-A-1 (left) and Eca-109 (right) cells detected by a laser scanning confocal microscope after treatment with (A) DMSO vehicle, (B) EGCG, (C) TF_3, (D) Vc, (E) EGCG + Vc and (F) TF_3 + Vc for 24 h. Highly condensed or fragmented nuclei represent apoptotic cells. Intact nuclei represent viable cells. (II) The proportion of apoptotic SPC-A-1 and Eca-109 cells treated by samples for 24 h. Significant differences between groups are indicated by different letters ($p < 0.05$).

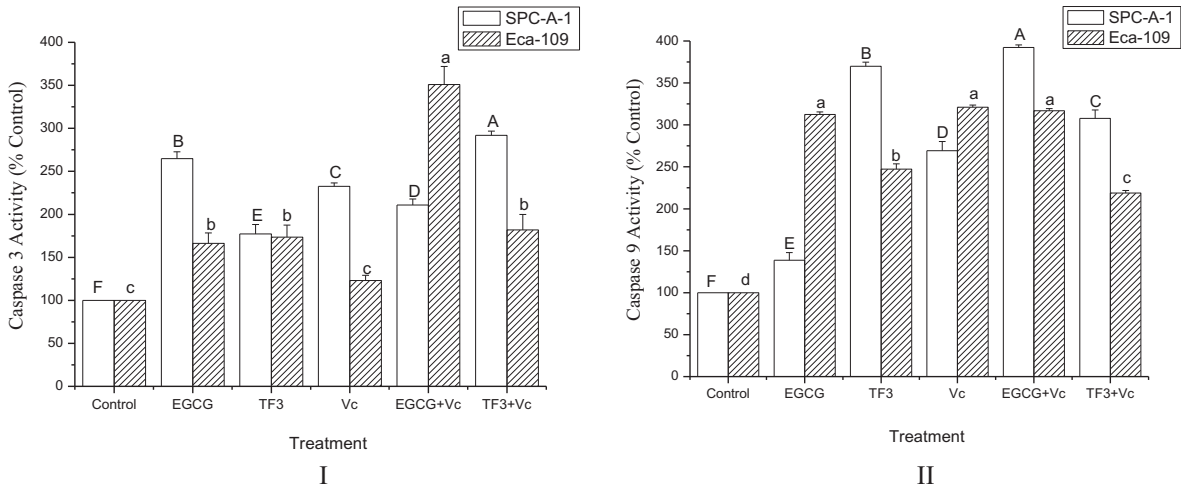

Fig. 2. EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc activate caspase-3/9 activity in SPC-A-1 and Eca-109 cells. Activities of caspase-3 (I) or caspase-9 (II) in SPC-A-1 and Eca-109 cells after treatment with DMSO vehicle, EGCG, TF_3, Vc, EGCG + Vc or TF_3 + Vc for 24 h. The concentration of each compound was 50 μmol/L. Significant differences between groups are indicated by different letters ($p < 0.05$).

concentration 50 μM of each compound for 24 h. After treatment, cells were resuspended with trypsin, lysated with lysis buffer on ice, and centrifuged at 16,000–20,000*g* for 10–15 min. The supernatant was collected, and the protein concentration of the supernatant was determined with Bradford method. The pNA absorbance at 405 nm was measured by a spectrophotometer (Lengguang model-752, Lengguang Optical Instrument Ltd. Co., Shanghai, China). The concentration of pNA/mg protein (caspase-3/9 activity) in samples was calculated according to the standard curve. Data was presented as means ± SD from 6 independent experiments.

2.5. Analysis of MAPK pathways

In the MAPK assay, synthetic ERK inhibitor PD98059, JNK inhibitor SP600125 and p38 inhibitor SB203580 were prepared in dimethyl sulfoxide (DMSO) at 20 μM, 10 μM and 20 μM, respectively. Cells were pretreated for 2 h with the mixture of three inhibitors or combination of any two of them, and then were treated with EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc (50 μM for each compounds) for 24 h. Apoptosis of SPC-A-1 cells and Eca-109 cells were then determined by Hoechst 33258 staining.

2.6. Statistical analysis

The data were presented as means ± standard deviations (SD). Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of <0.05 was considered significant. All computations were made by employing the SAS system for windows V8.

3. Results and discussion

3.1. Observation of cancer cell apoptosis

The inhibitory and pro-apoptotic effects of EGCG and TF_3 have been demonstrated in many cancer cell lines such as multiple lung cancer cells and human esophageal cancer KYSE 150 cells [16–18]. In this study, the changes of nuclear morphology of SPC-A-1 and Eca-109 cells with sample treatments for 24 h were analyzed under a laser scanning confocal microscope by Hoechst 33258 staining (Fig. 1). In the control group, the nuclei were stained less bright and looked uniform, indicating the cells were alive. In all treatment groups, numerous cells exhibited condensed or fragmented nuclei which were much brighter, implying that all samples could induce the apoptosis of SPC-A-1 and Eca-109 cancer cells (Fig. 1 I). The cells having a apoptotic body were counted and the percentage of apoptotic cells with EGCG + Vc and TF_3 + Vc treatment were significantly higher than that in single-compound treated groups ($p < 0.05$) for SPC-A-1 and Eca-109 cancer cells, indicating the addition of Vc could enhance the apoptotic induction of EGCG and TF_3 (Fig. 1 II). These results were consistent with the previous report that EGCG and Vc could synergistically inhibit liver cancer SMMC-7721 cell proliferation [19].

3.2. Enhanced activating effect of EGCG or TF_3 in combination with Vc on the caspase-3 and 9 activities

Caspases is a family of cysteine proteases which plays essential roles in apoptosis, necrosis and inflammation. Caspase-3 is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Caspase-9 is an initiator caspase, and has been linked to the mitochondrial death pathway. Stress signals causes release of cytochrome c from mitochondria and activation of apaf-1, which in turn cleaves the pro-enzyme of caspase-9 into the active form. Caspase-3 is then activated by caspases-9 through proteolytic cleavage. Sequential activation of caspase-3 plays a central role in the execution-phase of cell apoptosis [20].

In order to provide further assurance of the enhanced effects of EGCG or TF_3 in combination with Vc on apoptosis of SPC-A-1 and Eca-109 cells, the activities of caspase-3 and caspase-9 were measured in this work. Fig. 2 showed that the caspase-3 and caspase-9 activities of each treatment group were higher than that of the control group ($p < 0.05$), indicating EGCG, TF_3 and Vc induced apoptosis through the activation of caspase-3 and caspase-9. These results were consistent with the previous studies that EGCG and theaflavins could induce apoptosis via activating caspase-3 and caspase-9 in human lung cancer cells and leukemic monocyte lymphoma cells [21,22]. Moreover, for SPC-A-1 cells, the TF_3 + Vc treatment significantly increased the caspase-3 activity than the TF_3 or Vc treatment alone ($p < 0.05$), and the EGCG + Vc treatment obviously increased the caspase-9 activity than the EGCG or Vc treatment alone ($p < 0.05$). For Eca-109 cells, the combination of EGCG and Vc treatment significantly increased the activity of caspase-3 than

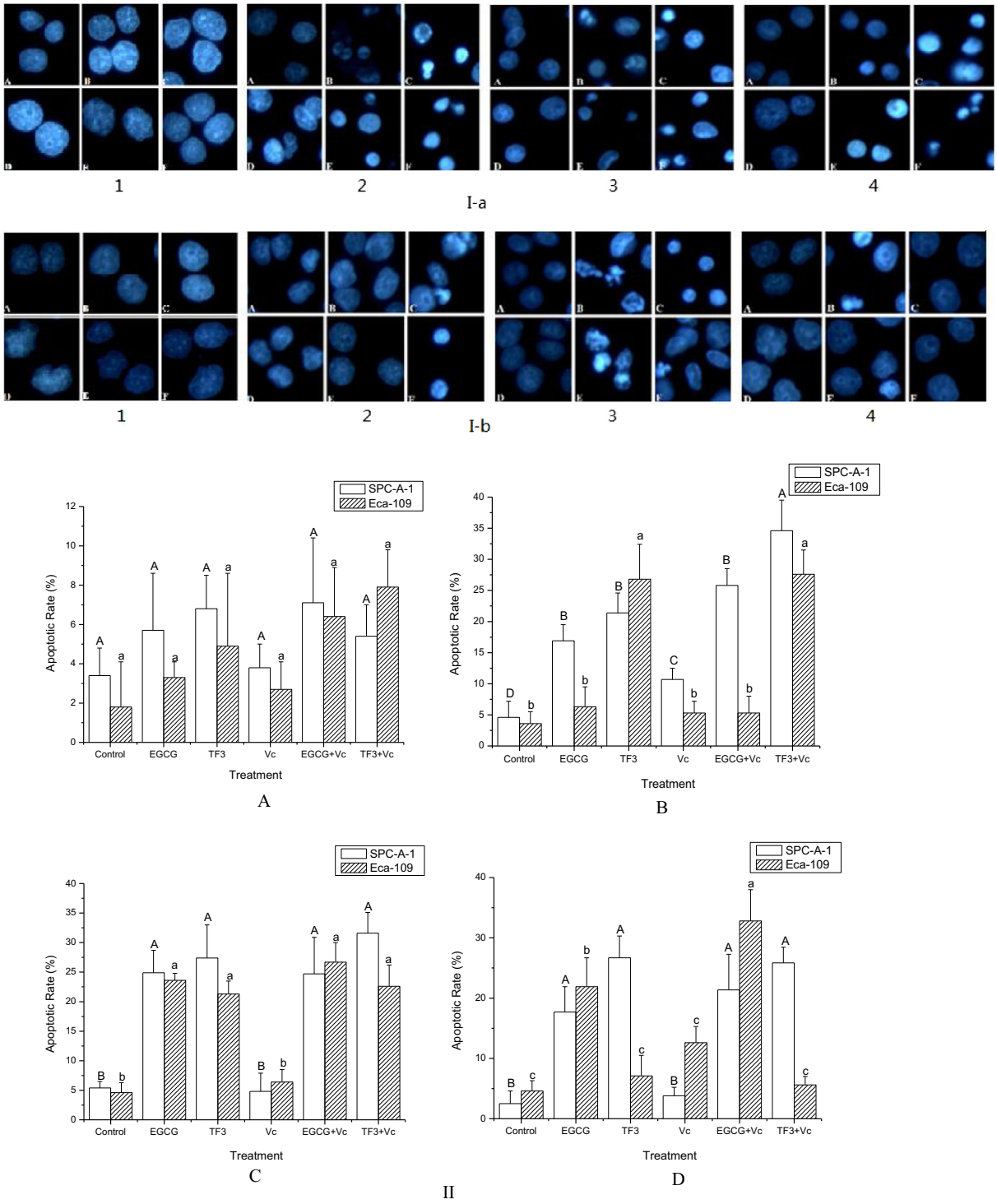

Fig. 3. The effect of EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc in inducing apoptosis of SPC-A-1 and Eca-109 cells was affected by pretreatment with different MAPK inhibitors. (I) Hoechst 33258 staining of SPC-A-1 (I-a) and Eca-109 (I-b) cells detected by a laser scanning confocal microscope after pretreatment with (1) JNK inhibitor + ERK inhibitor + p38 inhibitor, (2) JNK inhibitor + p38 inhibitor, (3) ERK inhibitor + p38 inhibitor, (4) JNK inhibitor + ERK inhibitor for 2 h and treatment with (A) DMSO vehicle, (B) EGCG, (C) TF_3, (D) Vc, (E) EGCG + Vc and (F) TF_3 + Vcfor 24 h. Highly condensed or fragmented nuclei represent apoptotic cells. Intact nuclei represent viable cells. (II) The proportion of apoptotic SPC-A-1 and Eca-109 cells pretreated with (A) JNK inhibitor + ERK inhibitor + p38 inhibitor, (B) JNK inhibitor + p38 inhibitor, (C) ERK inhibitor + p38 inhibitor, (D) JNK inhibitor + ERK inhibitor for 2 h and treated by samples for 24 h. Significant differences between groups are indicated by different letters ($p < 0.05$).

individual EGCG or Vc treatment ($p < 0.05$). These results demonstrated the enhancement of EGCG and TF_3 induced apoptosis by Vc in SPC-A-1 and Eca-109 cells involved the activation of the mitochondrial pathway with the increased activities of caspase-3 and 9.

3.3. EGCG or TF_3 with Vc induce apoptosis in SPC-A-1 and Eca-109 cells via the MAPK pathways

The MAPK pathways transduce a large variety of external signals, leading to a wide range of cellular responses, including

growth, differentiation, inflammation and apoptosis. In mammals, three major MAPK pathways have been identified: MAPK/ERK, SAPK/JNK, and p38 MAPK [23]. Chen et al. found EGCG activated all three MAPKs (ERK, JNK and p38) in a dose- and time-dependent manner in human hepatoma HepG2-C8 cells, and led to cell apoptosis [24]. Bhattacharya et al. revealed that theaflavins and thearubigins could lead to sustained activation of JNK and p38 but not ERK in A375 melanoma cells [23].

In order to explore whether the enhancement of EGCG and TF_3 induced apoptosis by Vc in SPC-A-1 and Eca-109 cells was through the MAPK pathways, three MAPKs inhibitors (ERK inhibitor PD98059, JNK inhibitor SP600125 and p38 inhibitor SB203580) were used in this study. Fig. 3 IIA showed when MAPK pathways were inhibited by the mixture of three inhibitors, the apoptotic rates of SPC-A-1 and Eca-109 cells treated by EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc were not significantly different from that of the controls ($p > 0.05$), indicating that EGCG, TF_3 and Vc induced apoptosis through MAPK pathways. When cells were pretreated with JNK and p38 inhibitors together (Fig. 3 IIB), EGCG, TF_3, Vc, EGCG + Vc and TF_3 + Vc all remarkably increased the apoptotic rates in SPC-A-1 cells ($p < 0.05$), while EGCG, Vc and EGCG + Vc did not induce the rise of apoptotic rates in Eca-109 cells ($p > 0.05$). When cells were pretreated with ERK and p38 inhibitors together (Fig. 3 IIC), all samples except for Vc significantly increased the apoptotic rates in both SPC-A-1 and Eca-109 cells ($p < 0.05$). After pretreated by the combination of ERK and JNK inhibitors (Fig. 3 IID), the apoptotic rates of SPC-A-1 cells were significantly increased by all samples except for Vc ($p < 0.05$), and the apoptotic rates of Eca-109 cells were obviously raised by EGCG, Vc and EGCG + Vc ($p < 0.05$).

Taken together, these results indicated EGCG, TF_3, EGCG + Vc and TF_3 + Vc induced the apoptosis of SPC-A-1 cells via ERK, JNK and p38 pathways, and Vc induced the apoptosis of SPC-A-1 cells via ERK pathways. For Eca-109 cells, TF_3 and TF_3 + Vc induced apoptosis via ERK and JNK pathways, EGCG and EGCG + Vc induced apoptosis via JNK and p38 pathways, and Vc only induced apoptosis via p38 pathways. The difference among signal pathways activated by EGCG, TF_3 and Vc may explain the enhancement of EGCG and TF_3 induced apoptosis by Vc in SPC-A-1 and Eca-109 cells.

In conclusion, Vc could enhance the EGCG and TF_3 induced apoptosis in SPC-A-1 and Eca-109 cells, and this effect involved the activation of caspase-3 and 9. EGCG, TF_3 and Vc could activate MAPK pathways respectively, and each compound activated different MAPK subfamilies in different cells. Our study here provides insights into the mechanism by which Vc can increased the tea polyphenols induced apoptosis in SPC-A-1 and Eca-109 cells. This will ultimately aid the design of more effective anti-cancer treatments.

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