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Combined Treatment of Sodium Salicylate and Genistein Induces Incomplete Apoptosis and Necrosis in MCF-7 Multicellular Tumor Spheroids

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Aspirin and its deacetylated form, sodium salicylate (NaSal), have been shown to exert chemopreventive activities against many human cancers including those of the colon, lung, and breast. Previously, we showed that combined treatment of NaSal and genistein synergistically induced apoptosis in A549 lung cancer cells, indicating that these two natural chemicals could be used in combination for cancer therapy. In this study, we examined effects of NaSal/genistein combined treatment on other cancer cells and in three-dimensional multicellular tumor spheroid (MTS) and in an *in vitro* solid tumor model. We found that the combined treatment induces apoptosis in the HCT116 cells and the A549 cells, but not in the MCF-7 cells. Interestingly, the MCF-7 cells responded to the NaSal/genistein combined treatment by undergoing cell death when they were cultivated as MTS. The combined treatment induced apoptosis at an earlier stage in the MCF-7 MTS culture. However, when the MCF-7 MTS was cultivated for a longer period, it induced necrosis rather than apoptosis. We further found that the apoptotic pattern observed in MCF-7 MTS was incomplete: the chromatins were condensed and fragmented, but the nuclear membrane was still intact. Taken together, these results demonstrate that the NaSal/genistein combined treatment induces incomplete apoptosis and necrosis in the MCF-7 MTS culture system.

Key words : Salicylate, genistein, apoptosis, necrosis, MCF-7 multicellular tumor spheroids

Introduction

Aspirin and its natural deacetylated form, sodium salicylate (NaSal), exert anti-inflammatory activities by repressing the expression of diverse proinflammatory molecules including prostaglandin [1,5,22]. These nonsteroidal anti-inflammatory drugs have been also shown to have tumor suppressive activities against a variety of human cancers including colon, lung, and breast cancers, and leukemia. Daily intake of >300 mg aspirin is recommended to reduce the risk of recurrent colorectal adenoma and cancer. NaSal induces either cell cycle arrest or apoptosis in a dose-dependent manner in many cancer cells [2,3,13-15,18]; high dose (20 mM) of NaSal induces apoptosis in A549 lung cancer cells, whereas low dose (2-10 mM) induces cell cycle arrest [15]. Caspase family including caspase-3 and -8 has been implicated in NaSal-induced apoptosis. Previously, we showed that low concentration (2 mM) of NaSal activated the pro-survival and anti-apoptotoic protein kinases Akt/PKB and ERK1/2 [12]. Inhibition of both the Akt/PKB and ERK1/2 signaling has been shown to increase the sensitivity of A549 cells to NaSal. Similar results were obtained when 2 mM NaSal was treated in combination with 100 μM genistein [12], which is an inhibitor of receptor tyrosine kinases (RTKs) that are upstream of PI3K-Akt/PKB and MEK1/2 signaling [4,6,24]. The findings suggested that these two natural chemicals could be used in combination for cancer therapy.

Multicellular tumor spheroids (MTS) closely resemble avascular regions of *in vivo* solid tumors [8,9]. Similar to *in vivo* tumors, MTS exhibit a proliferation gradient with an outer region of proliferating cells that surrounds an inner region of quiescent cells and a core region. The cells in the innermost region experience insufficient nutrient and oxygen supply and thus die in particular by necrosis, to form the necrotic core. In addition, cancer cells develop a resistance when grown in spheroids, referred to as 'multicellular resistance' (MCR), through diverse mechanisms inherent in the spheroid structure.

In this study, we examined the effects of NaSal/genis-

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tein-combined treatment in 2D culture and MTS system. Here we show that the combined treatment induces apoptosis in HCT116 cells as well as A549 cells. We also demonstrate that the combined treatment induced either incomplete apoptosis or necrosis depending on the stage of MCF-7 MTS culture.

Materials and Methods

Cell culture and drug treatment

Human breast cancer MCF-7, lung cancer A549, and colon cancer HCT116 cells were obtained from American Type Culture Collection and cultured as two dimensional (2D) monolayers in DMEM (WelGene) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin-streptomycin in a 37°C humidified incubator with 5% CO_2. The cells were pre-treated with genistein (Calbiochem, 100 μM), LY294002 (Sigma, 20 μM), or U0126 (Sigma, 20 μM) for 1 hr, and then treated with NaSal (Sigma, S-3007) for the indicated times in the presence of the inhibitors.

Multicellular tumor spheroid culture [10,11]

For 3D cell culture, MCF-7 cells (provided by Yook JI, Yonsei University) were seeded at a density of 400 cells in 200 μl medium into 1.2% agarose-precoated well of 96 well plates. After 3 days of culture, the spheroids were fed by carefully aspirating 100 μl of medium and replacing with the same quantity of fresh medium every other day.

Hoechst 33258 and propidium iodide (HO/PI) double staining

To determine the cell death mode, Hoechst 33258 (HO) and propidium iodide (PI) double staining was performed. In 2D culture, cells were seeded at a density of 2.5×10^5 cells/ml in 35-mm dishes. After 24 hr, the cells were treated with the indicated chemicals for the indicated times and then stained with HO (1 μg/ml) and PI (5 μg/ml) for 15 min. In 3D culture, equal numbers of spheroids were transferred to 1.2% agarose-coated 60-mm dishes. The spheroids were treated with the indicated chemicals for the indicated times and trypsinized and then stained with HO/PI.

MTS staining [10,11]

Equal numbers of spheroids were transferred to 1.2% agar-coated 60 mm dishes and pretreated with the inhibitors for 3 hr, and treated with NaSal for the indicated times. Spheroids were collected, washed with PBS, fixed in 1% paraformaldehyde in PBS overnight at 4°C, and dehydrated in a graded series of ethanol that were embedded in paraffin. After processing into paraffin blocks, 6 μm sections were cut and mounted on glass slides, deparaffinized, rehydrated, and stained with HO and PI as described above.

Results and Discussion

Combined treatment of sodium salicylate and genistein induces apoptosis in A549 and HCT116 cells, but not in MCF-7 cells

Previously, we observed a synergistic effect of combined treatment of NaSal and genistein to induce apoptosis in A549 lung cancer cells. In this study, we examined the effects of the combined treatment in other cancer cells. HO/PI double staining method was used to identify necrosis (a tumor-promoting cell death type) as well as apoptosis (a tumor-suppressive cell death type); HO penetrates non-selectively plasma membrane of both damaged and intact cells and binds to DNA, causing a blue nuclear fluorescence, while PI penetrates only cells with damaged-membranes, causing red nuclear fluorescence. Thus, the cell death mode could be discriminated morphologically by nuclear fluorescence images: intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei, and intact pink nuclei indicated viable, early apoptotic, late apoptotic (secondary necrotic), and necrotic cells, respectively. Late apoptosis is only detected in *in vitro* culture system because in *in vivo* system the cells undergoing late apoptosis are removed by macrophage-mediated phagocytosis. As demonstrated previously [12], when A549 cells were treated with 2 mM NaSal combined with 100 μM genistein for 48 hr, the apoptotic rate was dramatically increased up to 66% (Fig. 1). The treated A549 cells formed the typical apoptotic bodies with brightly stained heterogeneous masses of chromatin and disrupted nuclear membrane. Similar results were obtained in HCT116 cancer cells (Fig. 1). In contrast, the combined treatment did not induce apoptosis in MCF-7 breast cancer cells (Fig. 1). Thus, NaSal/genistein-combined treatment induces different responses (either to induce apoptosis or not) depending on the cell types.

NaSal/genistein-combined treatment induces incomplete apoptosis and necrosis in MCF-7 MTS culture system

We further examined the effects of combined treatment

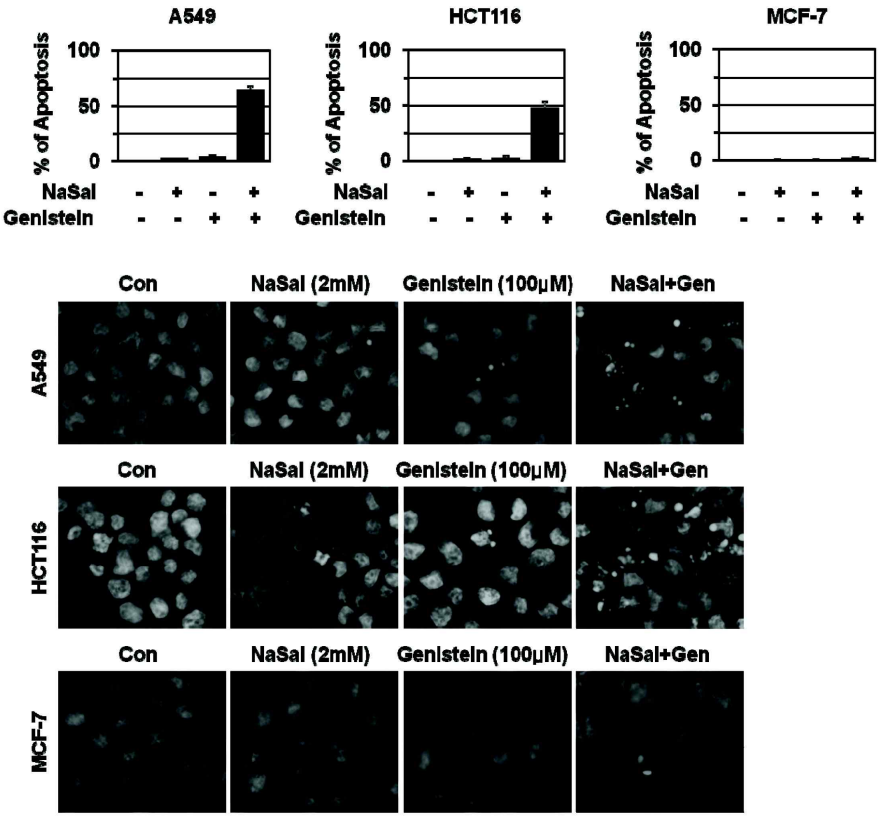

Fig. 1. NaSal/genistein-combined treatment induces apoptosis in A549 and HCT116 cells, but not in MCF-7 cells. A549, HCT116, and MCF-7 cells were cultured and treated with NaSal (2 mM) combined with genistein (100 μM) for 48 hr. Then they were stained with HO/PI and observed by fluorescence microscopy (lower panel) and apoptotic and necrotic cells were scored (upper panel). Results (500-800 cells in each group) are expressed as the means±SEM from three independent experiments.

of genistein and NaSal in MCF-7 MTS system as an *in vitro* solid tumor model [8,9]. MCF-7 cells were seeded at 400 cells per well into 96-well plates pre-coated with 1.2% agar. When seeded in non-adhesive conditions, the MCF-7 cells aggregated to form multicellular spheroids, of which disintegration required extended trypsinization and physical strength. The size of MCF-7 spheroids increased gradually and reached approximately 700 μm at 9 days [10,11]. In MTS, the cells in the core region experience limited oxygen and nutrient supply, thereby dying by necrosis to form the necrotic core when the spheroids reached more than 500 μm in diameters. The necrotic core formation was observed in the 8 day-cultured MCF-7 spheroids [10,11].

Interestingly, the combined treatment induced apoptosis in MCF-7 spheroids (Fig. 2) in contrast to monolayer MCF-7 culture (Fig. 1). In general, cancer cells exhibit MCR when grown in spheroids. Therefore, it is unexpected that the combined treatment induces apoptosis in MCF-7 MTS but not in monolayer-cultured MCF-7 cells. The underlying mechanism for this unusual phenomenon is not clear. Tumor microenvironmental factor such as hypoxia and necrotic core region may be associated with the alterations in the sensitivity of MCF-7 cells to combined treatment of NaSal and genistein. Spheroid-specific structure may be additionally linked to this unusual finding. The mechanism for this phenomenon remains to be elucidated in future. We further found that the apoptotic cell death was gradually reduced with developing MCF-7 MTS in culture (Fig. 2). The combined treatment caused approximately 20% of cells to undergo apoptosis at 4-5 days of MTS culture, whereas approximately 12% of cells to die by apoptosis at 6-7 days of MTS culture (Fig. 2). At 8 days of MTS culture in which the necrotic core was formed, only few cells (approximately 1-2%) appeared to undergo apoptosis in response to the combined treatment (Fig. 2). The findings suggested that the apoptotic resistance of MCF-7 spheroids might be linked to the formation of necrotic core. Interestingly, when MCF-7 MTS were cultivated for a longer period, the combined treatment

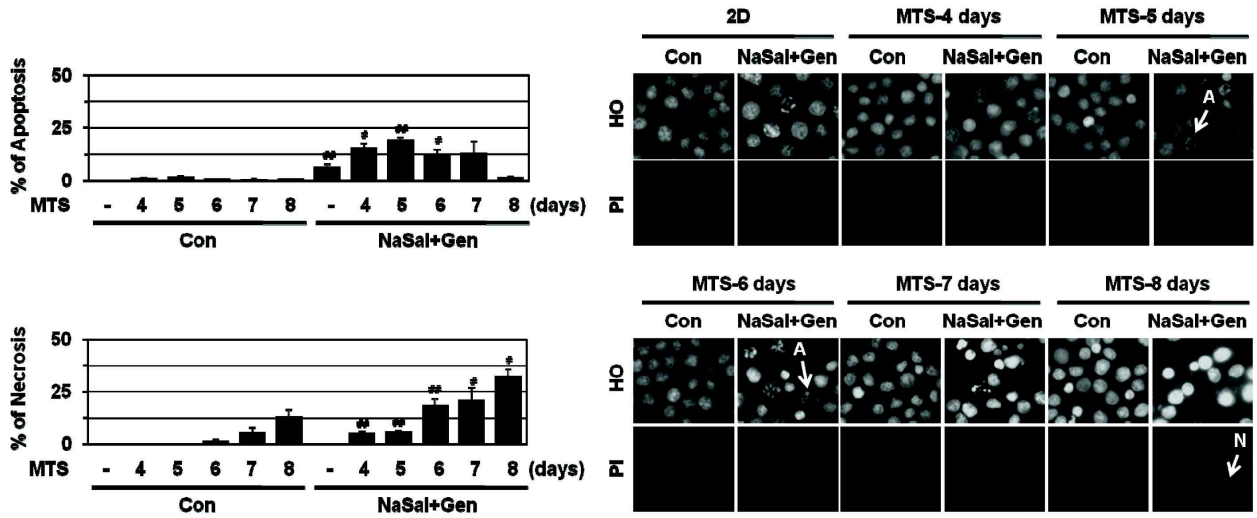

Fig. 2. Apoptotic and necrotic responses of MCF-7 spheroids to NaSal/genistein-combined treatment. MCF-7 cells were seeded into 1.2% agarose-coated 96-well plates at a density of 400 cells per well to form MTS. The MTSs were cultured for up to 8 days and treated with NaSal (2 mM) combined with genistein (100 μM) for 48 hr. MTS were dissociated into individual cells as described in 'Materials and methods' and stained with HO and PI and observed by fluorescence microscopy. Apoptotic and necrotic cells were scored. Results (500-800 cells in each group) are expressed as the means±SEM from three independent experiments. A, apoptotic cell; N, necrotic cell.

induced necrosis rather than apoptosis (Fig. 2). To confirm the MTS cultivation-dependent apoptosis-to-necrosis switch in response to the combined treatment, we assessed the apoptotic and necrotic cell death in the paraffin sections of spheroids, which were stained with HO and PI. As expected, the combined treatment predominantly induced apoptotic cell death at 6 days of MTS culture, but necrotic cell death at 8 days of MTS culture (Fig. 3). Thus, necrotic microenvironments may exert inhibitory effects on apoptotic stimuli-induced apoptosis and switch the cell death mode to necrosis.

In MCF-7 MTS, NaSal/genistein-combined treatment-induced apoptosis appeared to be incomplete; although the chromatins were condensed and fragmented, the nuclear membrane was intact and apoptotic bodies were not detected (Fig. 4A). In contrast, when A549 and HCT116 cells were treated with combination of NaSal and genistein, the nuclei were disrupted and fragmented into multiple pieces, to form the apoptotic bodies (Fig. 1). Apoptotic process had been classified four sequential stages (I-IV) according to the progression of chromatin condensation [19]. Nuclei of control cells are defined as stage I. Upon apoptotic stimuli, the chromatin began to condense against the nuclear periphery (stage II) and then condensed into discrete peripheral clumps (stage III). Eventually, the chromatin masses are aggregated to form discrete apoptotic bodies with loss of the nuclear membrane (stage IV). MCF-7 cells exposed to NaSal/genistein-combined treatment appeared to be blocked at an early stage of chromatin condensation (stage II). Interestingly, while discrete apoptotic bodies (stage IV) were detected in A549 cell exposed either to NaSal/genistein or to NaSal/U0126 (ERK1/2 inhibitor)/LY294002 (PI3K-Akt/PKB inhibitor), incomplete apoptosis (stage II) was observed in A549 cells treated with NaSal/U0126 (Fig. 4B). These results indicate that NaSal-activated ERK1/2 signaling may exert an inhibitory effect on the switch form stage I to stage II chromatin structure, whereas the PI3K-Akt/PKB pathway inhibit the conversion of stage II/III to stage IV chromatin structure.

Biological relevance of this study

In this study, we showed that while NaSal/genistein-combined treatment induced incomplete apoptosis at the earlier phase of MCF-7 MTS culture, it induced necrosis at the later phase of MTS culture. Necrosis is characterized by the cell membrane rupture to discharge the cellular contents including high-mobility group box 1 protein (HMGB1) into the extracellular space [7,17,20,21,23,25]. HMGB1 was originally identified as a structural co-factor critical for proper transcriptional regulation in cells, but when it is released

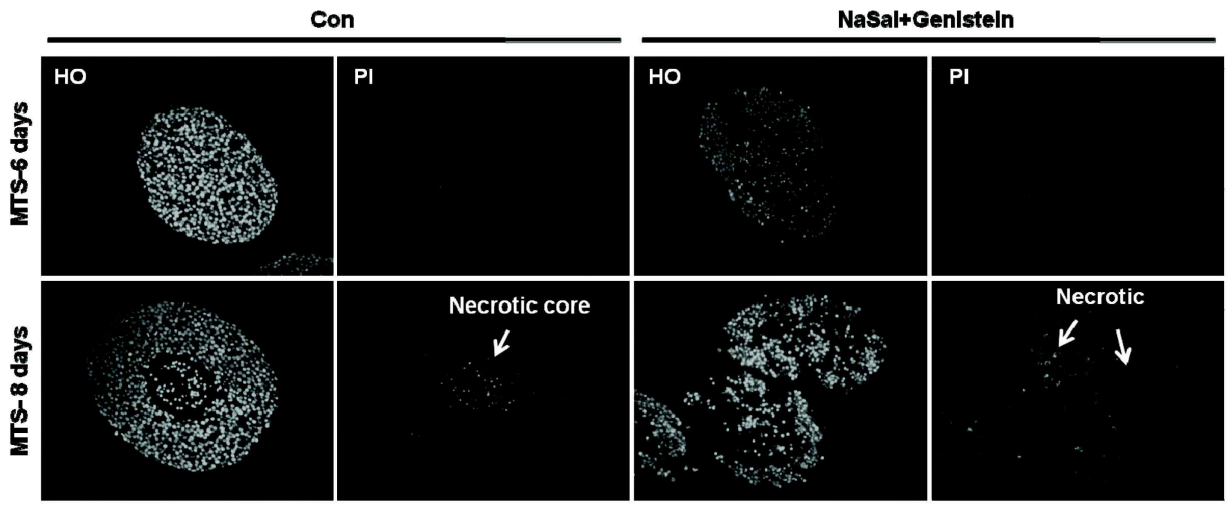

Fig. 3. MCF-7 spheroids were cultured for 6 or 8 days and were treated with NaSal (2 mM) in combination with genistein (100 μM) for 48 hr. The spheroids were harvested and fixed in 1% paraformaldehyde. After processing into paraffin blocks, the spheroids were sectioned. And the paraffin sections were stained with HO and PI.

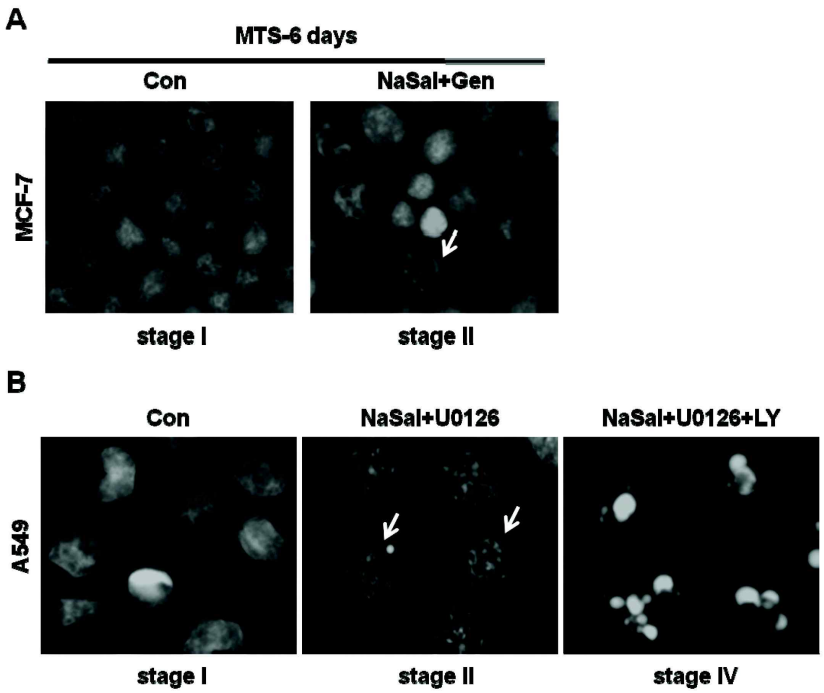

Fig. 4. (A) MCF-7 cells were seeded into 1.2% agarose-coated 96-well plates at a density of 400 cells per well and cultured for 6 days and were treated with NaSal (2 mM) combined with genistein (100 μM) for 48 hr. MTS were dissociated into individual cells as described in 'Materials and methods' and stained with HO and observed by fluorescence microscopy. (B) A549 cells were pretreated with U0126 (20 μM) and/or LY294002 (LY, 20 μM) for 1 hr and then treated with NaSal (2 mM) for 48 hr. Then the cells were stained with HO and observed with fluorescence microscopy. Arrow, incomplete apoptotic cell.

into the extracellular space, it acts as a cytokine to cause inflammatory response and to exert tumor-promoting and angiogenenic activities. Thus, necrosis is regarded as a cell death type that promotes tumor growth and angiogenesis by the action of HMGB1. HMGB1 also switches alkylating agent-induced apoptosis to necrosis [16]; thus, it may make tumor cells hyper-sensitive to anticancer drugs and to die by necrosis instead of apoptosis. In contrast, apoptosis is a

genetically regulated process of cell death that is characterized by membrane blebbing and DNA degradation. Eventually, apoptotic bodies are formed by cell disintegration and are removed by phagocytes or neighboring cells, resulting in the clean deletion of dead cells without inflammation. In this study, we found that the apoptotic pattern observed in MCF-7 MTS was incomplete; although the chromatins were condensed and fragmented, the nuclear membrane was intact. Because the apoptotic bodies were not found in the cells, we cannot exclude the possibility that the incomplete apoptotic cells may release cellular constituents such as HMGB1, thus evoking an inflammatory response. Based on these results, we suggest that caution should be given before the application of NaSal/genistein-combined treatment for cancer therapy. In particular, the strategy is needed to avoid undesirable necrotic death and incomplete apoptosis.

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초록 : MCF-7 MTS에서 sodium salicylate과 genistein 복합처리는 불완전한 세포사멸과 세포괴사를 유도한다.

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아스피린과 아스피린의 deacetylated form인 sodium salicylate (NaSal)은 대장암, 폐암 및 유방암을 비롯한 다양한 암의 항암제 활성을 나타내는 것으로 잘 알려져 있다. A549 폐암 세포주에 저농도의 NaSal과 genistein을 함께 복합 처리시 상승작용에 의해 세포사멸을 증가시켜서 NaSal에 의한 암억제 효과를 증대시킴을 이미 밝힌 바 있다. 본 연구에서는 A549가 아닌 다른 암세포주와 *in vitro* solid tumor model인 multicellular spheroids (MTS)을 이용하여 NaSal과 genistein 복합처리 효과를 조사하였다. NaSal/genistein 복합 처리시 A549 세포주와 마찬가지로 HCT116 세포주에서도 세포사멸이 유도되었지만, MCF-7 세포주에서는 유도되지 않았다. 흥미롭게도, MCF-7 세포주는 MTS로 배양되는 동안 NaSal/genistein 복합 처리에 의해 세포 죽음을 나타내었다. 세포 죽음의 형태는 MCF-7 MTS의 발달 단계에 따라 세포사멸 또는 세포괴사로 나타났다. MCF-7 MTS에서의 세포사멸은 불완전한 양상을 보였다. 즉 염색체가 응축되고 쪼개지지만, 핵막은 여전히 관찰되었다. 이상의 연구 결과 NaSal/genistein 복합처리는 MCF-7 MTS 배양 system에서 불완전한 세포사멸과 세포괴사를 일으킴을 알 수 있었다.