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Differential Effects of Grape Seed Extract against Human Colorectal Cancer Cell Lines: The Intricate Role of Death Receptors and Mitochondria

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Abstract

Failure of anti-cancer therapy in colorectal cancer (CRC) cells involves resistance to death mechanisms. We investigated grape seed extract (GSE) ability to target CRC cells and delineated the mechanisms involved in GSE-induced CRC cell death. GSE selectively induced apoptotic death in human CRC cells; efficacy increased as the metastatic potential of the cancer cells increased. Oxidative stress, loss of mitochondrial membrane potential, modulation of pro- and anti-apoptotic proteins, and involvement of both caspase-dependent / independent apoptotic pathways contributed to GSE-induced CRC cell death. GSE intervention may serve as a multitargeted CRC therapeutics, capable of inducing selective cancer cell death.

Keywords

grape seed extract; apoptosis; death receptors; colorectal cancer; mitochondrial membrane potential

1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths in the United States [1]. The incidence of CRC is also increasing world-wide, with an over-all lifetime risk of 1 in 19, due to adoption of western lifestyle habits; high fat diet, alcohol consumption, and sedentary lifestyle [2]. Compliance with screening recommendations is low; as such 60% of new CRC cases are diagnosed at a stage where they have already

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progressed to advanced disease [2]. Conventional therapies for CRC, e.g. chemotherapy, radiation, and surgery, result in severe toxicity and associated therapy resistance [3; 4]. Such limitations have resulted in a shift towards additional strategies involving the use of natural dietary/non-dietary products, which target multiple pathways in cancer cells and are associated with limited or no toxicity [5; 6].

In this regard, studies have indicated that high consumption of fruits and vegetables or their bio-active components can decrease CRC incidence by 40% [6; 7]. Grape Seed Extract (GSE) is one such widely consumed dietary supplement (with 95% standardized procyanidins) that has been shown to possess anti-cancer, anti-inflammatory, anti-oxidant, anti-bacterial, anti-viral effects [5; 8; 9; 10]. Pre-clinical studies have established strong GSE efficacy against prostate, colon, lung, breast, skin, and other cancers [5; 11; 12; 13; 14; 15; 16; 17; 18; 19]. Clinical studies with GSE have indicated that its active components are bioavailable and well tolerated [20; 21; 22]. However, till date, the specificity of GSE to target CRC cells and the associated-mechanisms involved in GSE-induced CRC cell death are not well studied. Therefore, in the present study, we investigated the molecular mechanisms involved in GSE-induced CRC cell death at various stages of the malignancy. Though it has been previously shown by a number of research groups, including ours, that GSE has the ability to induce CRC cell death in both *in vitro* and *in vivo* CRC models [5; 13; 14; 15; 18; 19; 23; 24; 25], one limitation of all the *in vitro* studies investigating GSE efficacy in CRC was that the *in-vitro* experimental design failed to take into consideration the different stages of this deadly malignancy as well as normal colon epithelial cells. Our present study is unique in that aspect, as we chose multiple CRC cell lines, which differ in their metastatic potential; this strategy helped in elucidating the differential effects of GSE against human CRC cell lines.

2. Materials and Methods

2.1 Reagents

Standardized preparation of GSE was a gift from Kikkoman Corp. (Nado City, Japan). The composition of the GSE preparation is listed as: 89.3% procyanidins, 6.6% monomeric flavonols, 2.24% moisture content, 1.06% of protein, and 0.8% of ash. Dimethyl Sulfoxide (DMSO) and N-acetyl cysteine (NAC) were from Sigma Chemical Co. (St. Louis, MO); Trypan blue 0.4% was from Invitrogen (Carlsbad, CA). Primary antibodies used were anti-cleaved caspase-9, anti-cleaved caspase -8, anti-cleaved caspase -3, anti-cleaved PARP, anti-COX IV, anti-AIF, anti-Bak, anti-Bik, anti-p53, anti-p21, and anti-Puma (Cell Signaling Technology, Beverly, MA); anti-DR4, anti-DR5, and anti-Mcl-1s (Santa Cruz Biotechnology, Santa Cruz, CA); anti-cytochrome-c, and anti-GADPH (BD Biosciences, San Jose, CA); and anti-β-actin (Sigma, St. Louis, MO). Anti-mouse and anti-rabbit horseradish peroxidase (HRP) secondary antibodies were purchased from Invitrogen and Cell Signaling Technology, respectively. Z-VAD-Fmk, DR4/Fc, and DR5/Fc were from R&D (Minneapolis, MN).

2.2 Cell lines

SW480 and HCT116 cells were purchased from American Type Culture Collection (Manassas, VA). SW620 cells were a gift from Dr. Pamela Rice, University of Colorado, Denver. NCM460 cells were from InCell Corporation (San Antonio, TX). HCT116 vector control and HCT116 p53 double knockout cells were a kind gift from Dr. Bert Vogelstein (The Johns Hopkins Kimmel Cancer Center). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere in DMEM (HCT116), Leibovitz-L15 (SW480), and RMPI (SW620) media supplemented with 10% FBS and 1% penicillin/streptomycin. NCM 460 cells were maintained under similar conditions in M3:10 media (InCell Corporation).

2.3 Assessment of Cell Viability and Apoptosis

Cells were plated at a density of 5,000/cm² in 60-mm culture plates under standard conditions and treated subsequently either with DMSO alone or with varying concentrations of GSE (0-100 µg/mL) in DMSO. At the end of desired treatment times (12-48 h), cells were harvested by brief trypsinization and counted using a hemocytometer. Trypan blue dye exclusion assay was used to differentiate between live and dead cells. The final concentration of DMSO in the culture medium during different treatments did not exceed 0.1% (v/v). To quantify GSE-induced apoptotic death, Annexin V and propidium iodide staining was done using Vybrant Apoptosis Assay kit 2 as per the manufacturer's protocol and the stained cells were analyzed by FACS analysis, utilizing the core service of the University of Colorado Cancer Center (Aurora, CO), in order to quantify the apoptotic cells. In inhibitor studies, CRC cells were pre-treated for 2h with all-caspases inhibitor z-VAD-fmk (50 µmol/mL), DR4/Fc (100 ng/mL), DR5/Fc (100 ng/mL) or a combination of both DR4Fc and DR5/Fc prior to GSE exposure. In the experiments with NAC, CRC cells were pretreated for 1h with NAC (10 mM), in pH-7.5 adjusted media, followed by exposure to GSE (20 or 50 µg/ml) for 12h. Mitochondrial and cytosolic fractions of CRC cells were prepared as described previously [26].

2.4 Western Immunoblotting

At the end of each treatment, cell lysates were prepared in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, 5 units/mL aprotinin]. Immunoblot analysis using equal amount of protein lysate per sample was done. Membranes were probed with desired primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualized by enhanced chemiluminescence detection system. Some blots were multiplexed or stripped and reprobed with different antibodies including those for loading control.

2.5 Determination of Mitochondrial Membrane Potential (Ψ_m)

Changes in Ψ_m were assessed using the cationic dye, DiOC₆(3) that accumulates in mitochondria with active membrane potential. Emission increases due to dye stacking. The stain intensity decreases when agents disrupt the Ψ_m . Following 3-24h GSE treatment (20 and 50µg/ml), cells were exposed to 40nM DiOC6(3) for 20 min at 37 C and then cells were washed with fresh medium and fluorescence visualized at 488 nm using the live imaging

equipment: Operetta High-Content Imaging and Analysis System. The median values of green fluorescence, from 9 different focal points within the well, were determined with Harmony software. This live-imaging method allows for the quantification of cells with depolarized mitochondria.

2.6 Statistical Analysis

All the data shown are mean SE, representative of at least three independent experiments. Statistical significance of differences between control and GSE-treated samples were calculated by one-way ANOVA (Sigma Stat 2.03, Jandel Scientific, San Rafael, CA). *P* values of < 0.05 were considered significant.

3. Results

3.1 GSE treatment causes growth inhibition and induces death in human CRC cells

First, we evaluated the efficacy of GSE against a panel of human CRC cell lines, based on phenotypic and genetic variations, so as to cover different clinical stages of CRC, viz., SW480 (stage II CRC with mutant *p53*), SW620 (stage III CRC with mutant *p53*), HCT116 (stage IV CRC with wt *p53*) cells. Trypan blue exclusion assays were performed to differentiate between live and dead cells. Results indicated that GSE at 25, 50 and 100 µg/ml doses resulted in a dose-dependent growth inhibition of SW480 cells after 12, 24 and 48 h treatment; concomitantly, an increase in SW480 dead cell population [17-37% (*P*<0.05-0.001), 19-38% (*P*<0.01-0.001) and 15-60% (*P*<0.01-0.001)] was also observed under the same concentrations and treatment conditions (Fig. 1A). In case of SW620 and HCT116 cells (Fig. 1B & C), GSE showed significant effect on growth inhibition at comparatively lower doses (10-30 µg/ml) to those effective (25-100 µg/ml) in SW480 cells. In SW620 and HCT116 cells, these low GSE doses were also effective in causing significant cell death [50-60% (*P*<0.01-0.001)] in as early as 12 h, though the dead cell numbers did not increase further at later time points. Overall, GSE (10-100 µg/ml) treatment for 12-48 h significantly suppressed cell growth and induced cell death in a dose- dependent manner in the cell lines studied. Notably, since these CRC cells represent different stages of CRC, our findings showing stronger GSE inhibitory effects in cell lines that are more aggressive in nature have significant translational implications in CRC management. In addition to the viability studies, we also examined the GSE effect on cell-cycle distribution of CRC cells. GSE arrested SW480 cells in G2M phase, SW620 in the G1 phase, and HCT116, partly, in G1 and G2 phases (data not shown). Since the predominant effect of GSE in these CRC cells was cell death, we chose to focus the mechanistic studies on this aspect of GSE effect.

3.2 GSE treatment activates both the intrinsic and extrinsic apoptotic pathways in human CRC cells

Next, to identify the nature of GSE-mediated CRC cell death, quantitative apoptosis assay with AnnexinV/ Propidium iodide (PI) followed by FACS analysis was performed. GSE treatment for 12h induced strong apoptotic death, causing a 1.5 - 3 fold (*P*<0.05-*P*<0.001) increase in apoptotic SW480 cells at 25-100µg/mL doses, respectively (Fig. 2A). Unlike SW480 cells, a much lower GSE concentration was effective in inducing a apoptosis in the more metastatic cells lines, SW620 and HCT116, based on their cell death effects with GSE

in Fig 1. In these cell lines, significant apoptotic death (11 folds, $P<0.001$; Fig. 2A) was observed which remained consistent across different doses. Based on these results, showing strong apoptotic effect of GSE in a panel of human CRC cell lines, we next assessed the involvement of both extrinsic and intrinsic pathways in GSE-caused apoptotic death of these CRC cell lines. In all cell lines, GSE treatment induced cleavage of caspases 8, -9, -3 and PARP, suggesting the involvement of different executioner pathways in GSE induced apoptotic death (Fig. 2B). A time course assessment of cytochrome c (*cyto c*) release in the cytoplasm of GSE treated CRC cells indicated that *cyto c* was released within 9h in SW480 and SW620 cells while in HCT116 cells the release was seen after 12h (Fig. 2C).

Since reduction of Ψ_m is believed to be an early event during apoptosis [27; 28], to further solidify above findings, we examined the kinetics of the Ψ_m in GSE-treated CRC cells. Live fluorescence (Operetta imaging) using the Cationic dye DiOC₆(3) as an indicator of dissipation of Ψ_m revealed a time-dependent decay (Fig. 3) of DiOC₆(3)-fluorescence (indicating a decline in Ψ_m). In SW480 and SW620 cells the decline in DiOC₆(3)-fluorescence was seen as early as 3h, which became more significant by 12h. While in HCT116 cells the decline in Ψ_m was not significant by 3h but became strongly significant by 9h.

3.3 GSE treatment also induces caspase-independent intrinsic apoptotic pathway in human CRC cells and causes differential modulation of apoptotic proteins

Mutations within the caspase protease family are commonly observed in malignancies [29; 30; 31; 32; 33]. Several reports indicate caspase-8 mutations in breast and gastric cancers, while silent mutations of caspase-9 have been reported in CRC [29; 30; 31; 32; 33]. Therefore, for an effective clinical CRC therapy, the treatment agents also need to circumvent such genetic variations/limitations and display potential to induce caspase-independent apoptotic death in the cancer cells. In this context, we next examined whether GSE also had the potential to induce caspase-independent apoptotic death in the CRC cell lines. CRC cells were pre-treated with Z-VAD-FMK, an irreversible pan-caspase inhibitor, prior to 12h GSE treatment (20-50 μ g/mL) and apoptotic death was assessed (Fig. 4A). Importantly, we observed that the apoptosis induced by GSE in all three CRC cell lines was either unaffected or marginally decreased in presence of pan-caspase inhibitors. Thus, regardless of caspase inactivation, GSE was able to induce significant apoptotic death in all CRC cell lines.

Since a group of pro-apoptotic proteins such as AIF and Endonuclease G (Endo G), involved in caspases independent apoptotic cell death, are also released from mitochondria, and on translocation to the nucleus initiate DNA fragmentation and chromatin condensation [28], we next examined their involvement in GSE caused apoptotic death. Subsequent to GSE treatments, time-course evaluation of cytosolic fractions of CRC cells showed that in SW620 and HCT116 cells, AIF was released within 12h, while it was only released by 24 h in SW480 cells, indicating that indeed AIF was also involved in apoptotic induction by GSE (Fig. 4B). In other study, while expression of Endo G showed a marginal increase in SW480 cells after GSE exposure, its involvement in GSE-caused apoptosis of SW620 and HCT116 cells was not significant (data not shown).

Next, we analyzed the ability of GSE to modulate a group of additional pro- and anti-apoptotic proteins that play an essential role in the extrinsic and intrinsic apoptotic pathways (Fig. 4C). Results showed that GSE treatment caused an up regulation of Death receptors, DR4 and DR5 in SW480 and SW620 cells, while only DR5 was specifically up regulated in HCT116 cells. Furthermore, the expression of pro-apoptotic proteins Mcl-1s and Bik was increased by GSE in all three CRC cell lines (Fig. 4C); however, there was down regulation of anti-apoptotic proteins Bcl-2 and XIAP by GSE only in SW480 cells (data not shown). Due to genetic variations within different CRC cell lines, SW480 and SW620 cells harbor mutant but HCT116 with wild-type p53, we also assessed GSE effect on p53 and its transcriptionally activated targets, which could play an important role in both cell cycle progression and apoptosis. Importantly, GSE caused an up regulation of p53 expression together with an increase in the levels of p21, Puma and Bak in HCT116 cells (Fig. 4C), and only an increase in p21 in SW620 cells (Fig. 4C).

3.4 Apoptotic death induced by GSE in human CRC cells is mediated by Death receptors and oxidative stress

To further examine the involvement of Death receptors in the GSE induced apoptotic cell death in CRC cell lines, cells were pretreated with DR4/Fc and/or DR5/Fc chimeric proteins so as to inhibit the extrinsic apoptotic signals prior to GSE exposure (20-50 μ g/mL for 12h) and then assessed for apoptotic death (Fig. 5A). In SW480 cells, pre-treatment with a single chimera protein DR4/Fc or DR5/Fc or a combination of both chimeras resulted in a marginal to significant attenuation of GSE-induced apoptotic death; the combination showing significantly more decrease (50%) in apoptotic cells than the single chimeras alone (Fig. 5A) suggesting an overlapping involvement of DR4 and DR5 in GSE-induced apoptotic death of SW480 cells. In SW620 cell line, however, only DR5/Fc chimera was able to marginally decrease the GSE-induced apoptotic death; the combination of both chimeras together not being more potent than the single DR5/Fc chimera alone (Fig. 5A). In HCT116 cells, DR5/Fc chimera alone was effective in significantly decreasing GSE induced apoptotic death, the combination of both chimeras failed to enhance the protective anti-apoptotic effect of DR5/Fc, suggesting the involvement of only DR5 in GSE-induced apoptotic death of HCT116 cells (Fig. 5A).

Considering the dependence of GSE-mediated cell death on the Death receptor pathway, we next focused on the upstream receptor stimulus. Several reports have indicated that proteasomal inhibition can lead to Death receptor up regulation, and protein accumulation of pro-apoptotic molecules namely Bik, Bim and Mcl-1 [34; 35]. To examine this possibility, we determined the efficacy of GSE to inhibit proteasomal activity directly in a cell free system (co – incubation with 20S proteasomal subunit) as well as in the cellular system (exposure of cells to GSE). Interestingly, GSE was able to inhibit the proteasomal activity of 20S subunit in a cell free system more significantly than epoxomicin, a known proteasomal inhibitor. However, GSE displayed no such effect in presence of cellular environment (data not shown), suggesting that other triggers were involved in causing the increase in the expression of the molecules involved in apoptosis.

We next focused on an alternative upstream stimulus, oxidative stress that has been implicated in Death receptor activation, mitochondrial DNA damage, and apoptosis cascade initiation [36; 37; 38] for its possible role in GSE mediated apoptosis. For this, CRC cells were pre-incubated with the anti-oxidant NAC prior to GSE (20-50 μ g/mL for 12h) exposure (Fig. 5B). Interestingly, NAC pretreatment resulted in 25% ($P<0.01$), 65% ($P<0.001$), and 37% ($P<0.001$) attenuation in GSE-caused apoptotic cell death in SW480, SW620, and HCT116 cells, respectively (Fig. 5B). Following these results, we also performed immunoblotting to visualize the changes in the protein expression of these Death receptor molecules resulting from NAC pre-treatment (Fig. 5C). The results obtained complimented the earlier observations using the chimera proteins in the receptor inhibition studies, i.e., in SW480 cells NAC pre-treatment resulted in down-regulation of both DR4 and DR5 receptors, while in SW620 cells no attenuation in the levels of death receptors was observed, in fact the expressions were slightly more in the NAC+GSE groups (Fig. 5C). Furthermore, we also observed down-regulation of DR5 protein expression levels with NAC pre-treatment in HCT116 cells, while there was no effect on DR4 levels. Overall, these results indicated that GSE-induced apoptotic death in SW480 and HCT116 cells is mediated *via* the extrinsic pathway with oxidative stress as a possible upstream stimulus. However, in SW620 cells, GSE caused apoptotic response, had minimal dependency on the extrinsic pathway. There was also significant attenuation in GSE induced apoptotic response in SW620 cells after NAC pre-treatment, suggesting that oxidative stress was a stimulus for GSE caused CRC cell death independent of Death receptors involvement.

3.5 GSE-mediated death is specific to CRC cells and is independent of their p53 status

To investigate whether the GSE induced cell death effects were specific to CRC cells and that it had no effect on normal colon cells, NCM 460 (normal colon epithelial cell line) cells were exposed to different doses of GSE (25-100 μ g/mL) for 12-48h (Fig. 6A) and cellular viability was assessed by Trypan blue exclusion assay. Importantly, no growth inhibition was observed in these normal colon cells due to GSE treatment. There was also no effect on cellular viability till 24h of GSE exposure, however, a marginal increase (8%) in dead cell population was observed after 48h exposure to the highest dose (100 μ g/mL) of GSE (Fig. 6A).

In other studies, based on the fact that there was a strong increase in p53 and its transcriptionally activated targets such as p21, Puma and Bak in HCT116 cells after GSE treatment, we further explored the role of p53 in GSE-induced CRC cell death. HCT116 p53 double knockout cells (HCT116 p53 KO) and vector control cells (HCT116 VC) were treated with GSE (10-30 μ g/mL) for 24h; viability assays (Fig. 6B & C) showed almost comparable GSE responses in both p53 KO and VC cells. Furthermore, in both cell lines we observed an up regulation of cleaved caspase-3, -9, -8, and cleaved PARP expression levels with GSE treatment, indicating that the apoptotic response induced by GSE was independent of p53 status (Fig. 6D).

4. Discussion

Mechanisms that contribute to multi-drug tumor cell resistance are known to involve mutations that result in uncontrolled cellular proliferation and apoptosis resistance; therefore, strategies that focus on inhibiting these processes, at any stage of malignancy, would be of clinical significance [39]. The process of apoptosis is highly complex involving a cascade of molecular events; the two main pathways involved in this process are: extrinsic or death receptor pathway and the intrinsic or mitochondrial-derived pathway [28]. There is evidence of cross-talk between these pathways and both pathways converge into the same executioner pathway [40]. The extrinsic pathway involves transmembrane receptor-mediated protein interactions. These death receptors are part of the tumor necrosis factor (TNF) receptor gene super family, and include DR4 and DR5 [28]. Ligand binding, for example Tumor Necrosis factor-related apoptosis-inducing ligand (TRAIL) and adaptor protein recruitment, such as Fas associated death domain (FADD), allow for the formation of the death-inducing signaling complex (DISC), which results in autocatalytic cleavage/activation of procaspase-8 and executioner pathway activation [41]. The intrinsic pathway, on the other hand, involves non-receptor mediated protein interactions and mitochondrial initiated events [28]. The stimuli that initiate the intrinsic pathways can be either positive or negative; negative signals involve the absence of certain growth hormones and cytokines, and positive signals include radiation, hypoxia, and presence of free radicals [28]. These stimuli ultimately alter Ψ_m resulting in the opening of the mitochondrial permeability transition pore. Loss of this membrane potential results in the release of pro-apoptotic proteins into the cytosol and executioner pathway activation [28]. Regulation of these mitochondrial apoptotic events occurs via members of the Bcl-2 family of proteins; these proteins can have either a pro-apoptotic or anti-apoptotic effect: anti-apoptotic molecules include: Bcl-2, Mcl-1L, and Bcl-xL; pro-apoptotic proteins include: Bik, Bim, Bak, Puma, and Mcl-1s [42]. Another major player in apoptosis is the p53 tumor suppressor molecule; p53-dependent cell death is executed by transcriptional activation of pro-apoptotic family members such as p21, Puma, Noxa, and Bak which can translocate to the mitochondria and induce cytochrome c release and result in executioner pathway activation [43; 44].

Clinically, a number of cancer types have been shown to be sensitive to TRAIL gene therapy resulting in the activation of the extrinsic pathway and ultimately cancer cell death [45; 46; 47]. However, resistance to TRAIL-mediated apoptosis in cancer cells has become a challenging issue in the clinic, with a number of cancer types, including CRC, exhibiting no response to therapy [48; 49; 50; 51]. Furthermore, mutations within the family of caspase proteases have also been reported in CRC, suggesting that effective clinical therapies are needed to circumvent these obstacles [31; 33].

In this regard, the results of the present study are highly significant as they identify GSE as a potential anti-cancer therapeutic agent that has the capacity to induce both extrinsic and intrinsic apoptotic pathways selectively in CRC, but not normal colon epithelial, cells. Previous studies have indicated caspase-dependent and caspase-independent pathways in response to GSE treatment, however this effect was GSE preparation specific, only examined a single non-metastatic colon cancer cell line, and did not utilize caspase pathway inhibition to determine dependence [52]. In this regard our study was designed to determine

GSE caspase dependence in various stages of CRC through irreversible pathway inhibition and results indicated that indeed GSE induced apoptotic cell death is not entirely caspase-dependent but involves the activation of caspase-independent pathways, which is mediated in part through the release of AIF. In addition, the GSE-mediated cell death is also dependent, in part, on the activation of the extrinsic pathway *via* death receptor up regulation in SW480 and HCT116 cells; SW620 cells failed to show any involvement of death receptors in GSE caused cell death. Interestingly, oxidative stress generated by GSE exposure was identified to play a role in the cell death of all CRC cell lines, as an upstream stimulus, causing either up regulation of death receptors (SW480 and HCT 116 cells) or generating cytotoxic stress (SW620 cells) affecting the mitochondrial membrane permeability. The upstream stimulus of oxidative stress not being able to cause significant death receptor up regulation, yet causing a significant apoptotic response in SW620 cells can be explained by the fact that in this cell line we observed p21 up regulation independent of p53 protein activation; previous work has linked GSE generated oxidative stress to p21 expression which leads to increased apoptotic response. Furthermore, our results indicate that loss of Ψ_m occurs prior to the release of *cyto c* followed by activation of intrinsic and extrinsic pathways, suggesting that the intrinsic pathway may be the initiating pathway in GSE-mediated apoptotic cell death. The GSE mediated apoptotic events were also found to be associated with differential modulation of pro- and anti-apoptotic proteins. In addition, the cytotoxic effect exhibited by GSE against CRC cells was found to be independent of p53 status of the CRC cell lines, though, it did induce the p53-dependent apoptotic pathway in HCT116, through up regulation of p53, Bak, p21, and Puma. This observation is highly significant, given the fact that one of the common mutations in CRC is missense mutations of the *TP53* gene, which encodes for the p53 protein [53].

Importantly, GSE mediated cell death efficacy was found to be specific against CRC cells as it exhibited no toxicity in normal colon epithelial cells. The fact that severe toxicity can result from conventional CRC therapies, for example, it took a long time to establish an optimal 5-FU regime that had less severe toxicity, higher response rate, and overall improved long-term survival [54], further reaffirms the benefits of GSE use. Another most significant finding of the present study is that GSE showed cytotoxic efficacy against different CRC cell lines, which differed in their metastatic potential; interestingly, the anti-cancer efficacy of GSE increased as the metastatic potential of CRC cells increased. Based on these observations, we can speculate that GSE intervention may serve as an effective, multi- target, non-toxic CRC therapy for the control of CRC at any stage of the malignancy.

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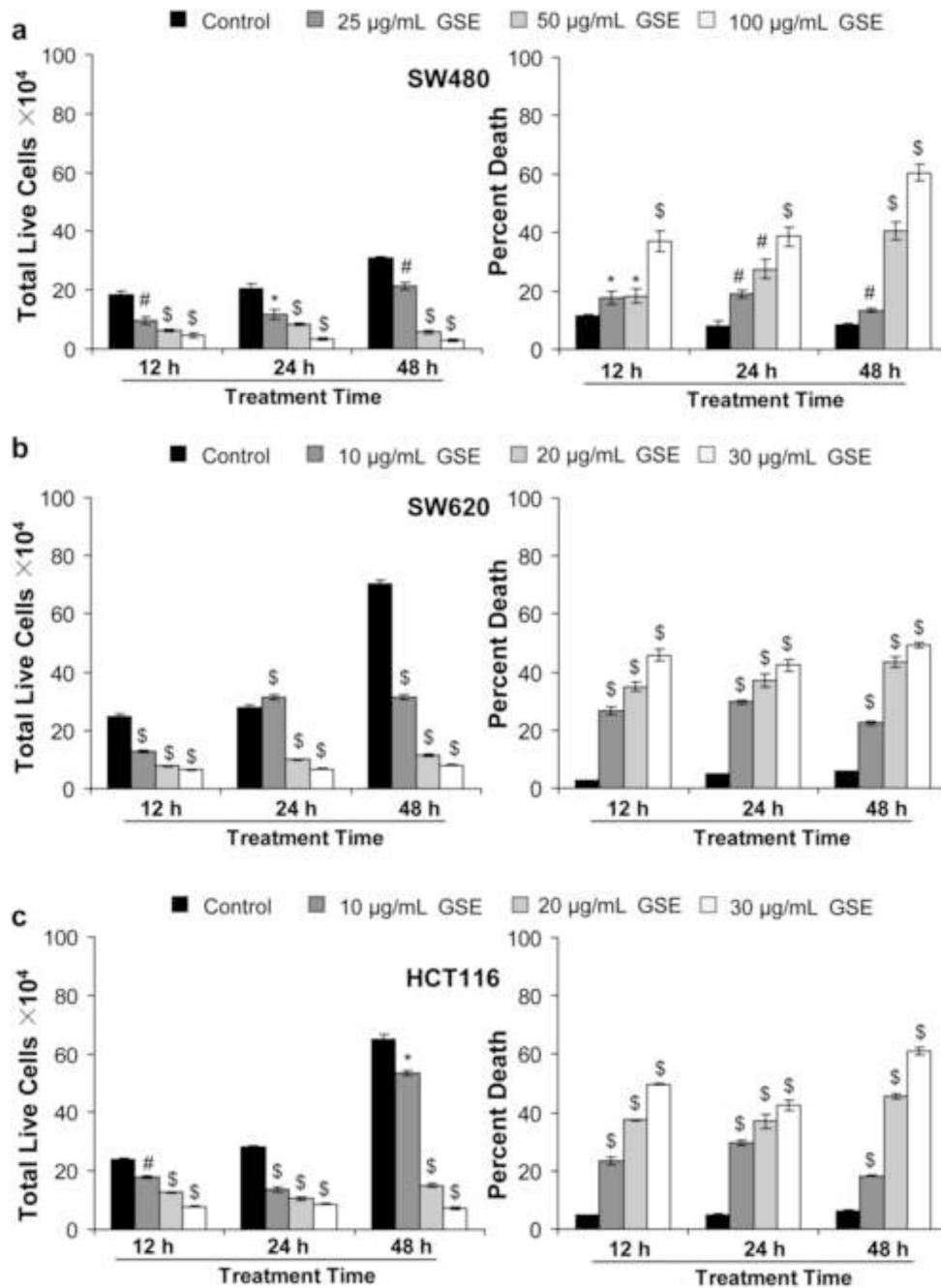


Figure 1. GSE treatment results in growth inhibition and death of human CRC cells

The biological activity of Kikkoman GSE was assessed in terms of its effect on cellular proliferation and viability of human CRC cells, measured as total live cell number and percent dead population after GSE exposure in A) SW480, B) SW620, and C) HCT116 CRC cells. GSE doses ranged from 10-100 µg/mL, and effect was observed for 12-48 hrs. The data shown here is representative of the mean of 3 independent values; bars, S.E.M.; * P 0.05; # P 0.01; \$ P 0.001; control (DMSO). S.E.M., standard error mean.

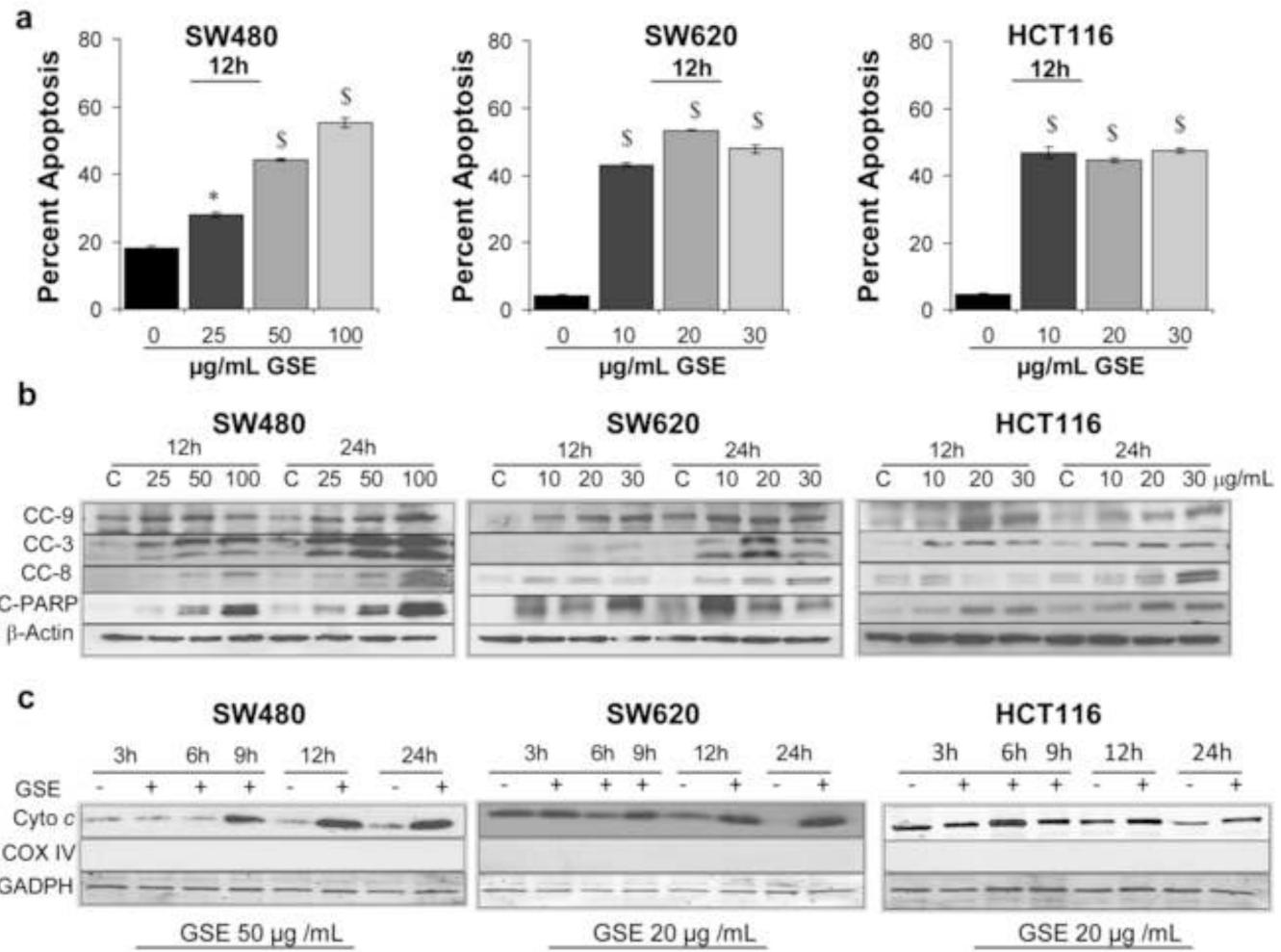


Figure 2. GSE treatment induces apoptotic death in human CRC cells

A) GSE causes apoptotic death of SW480, SW620, and HCT116 cells. Cells were treated with DMSO (control) or different concentrations of GSE (10-100 μg/mL) for 12 hours. At the end of treatment, cells were collected and stained with Annexin V-propidium iodide and analyzed by flow cytometry. % apoptotic death of CRC cell is represented by columns (mean values of three independent samples for each treatment ± S.E.M) * P 0.05; \$ P 0.001.

B) Apoptotic effect of GSE involves cleavage of caspases-9, -8, -3, and PARP. Both adherent and non-adherent cells were harvested and cell lysates were prepared in nondenaturing lysis buffer and immunoblotting was performed as detailed in Materials and Methods section. **C**) Apoptotic effect of GSE is accompanied with the release of cytochrome c (*cyto c*) in the cytoplasm of CRC cells. Membranes were also stripped and reprobed with anti-GADPH antibody to confirm equal protein loading. Probing with COX IV antibody revealed that the cytosolic fractions were non-contaminated with mitochondrial fractions. S.E.M, standard error mean.

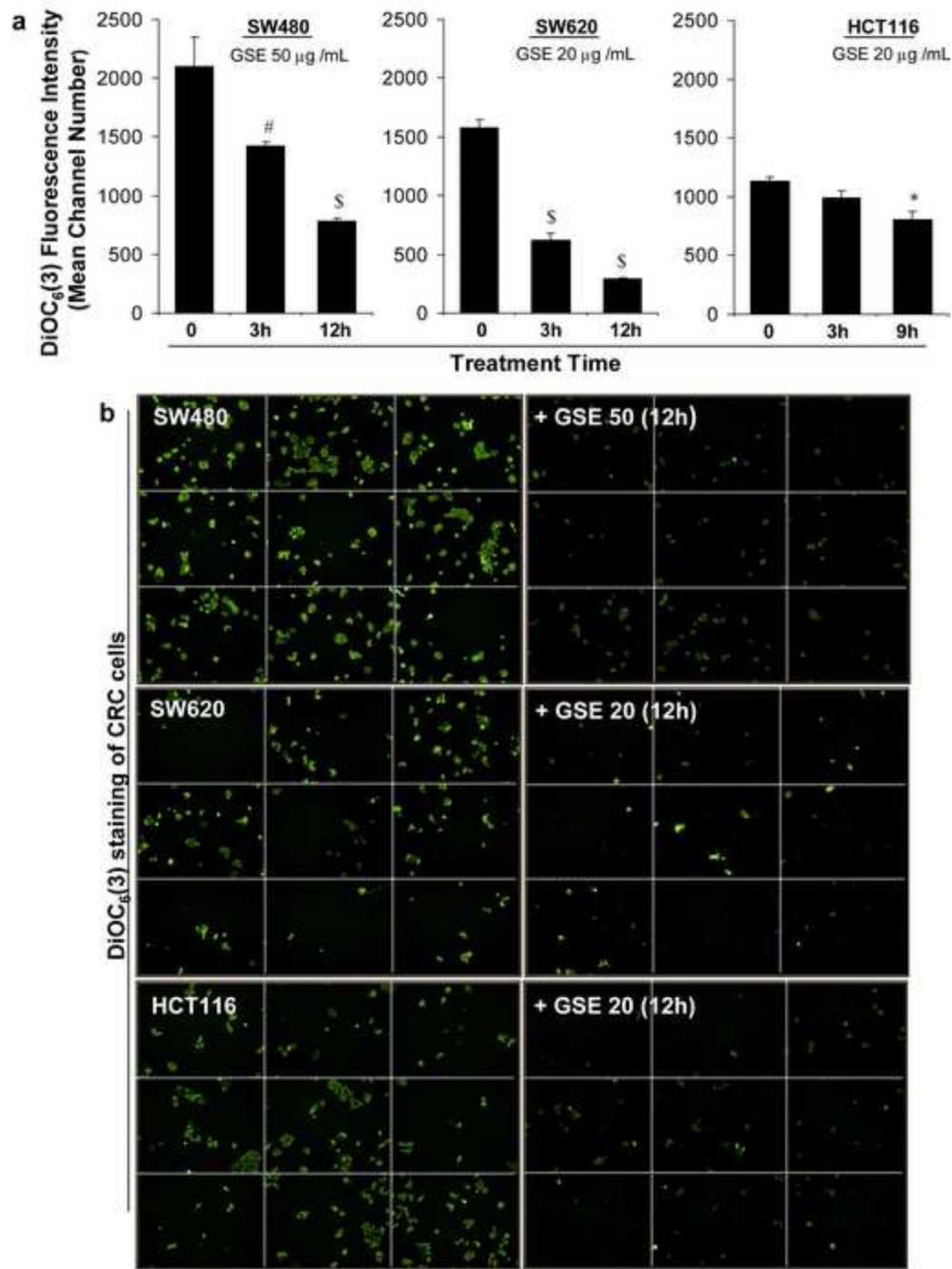


Figure 3. GSE treatment causes loss of mitochondrial membrane potential in human CRC cells
 Cells were exposed to 40nM DiOC₆(3) for 20 min at 37 C and then cells were washed with fresh medium and fluorescence visualized at 488 nm using the live imaging equipment: Operetta High-Content Imaging and Analysis System. **A**) The median values of green fluorescence, from 9 different focal points within the well, were determined with Harmony software and are represented as columns \pm S.E.M; * P 0.05; # P 0.01; \$ P 0.001. **B**) Representative photographs (20 \times) of 9 different focal points per well are shown. S.E.M, standard error mean.

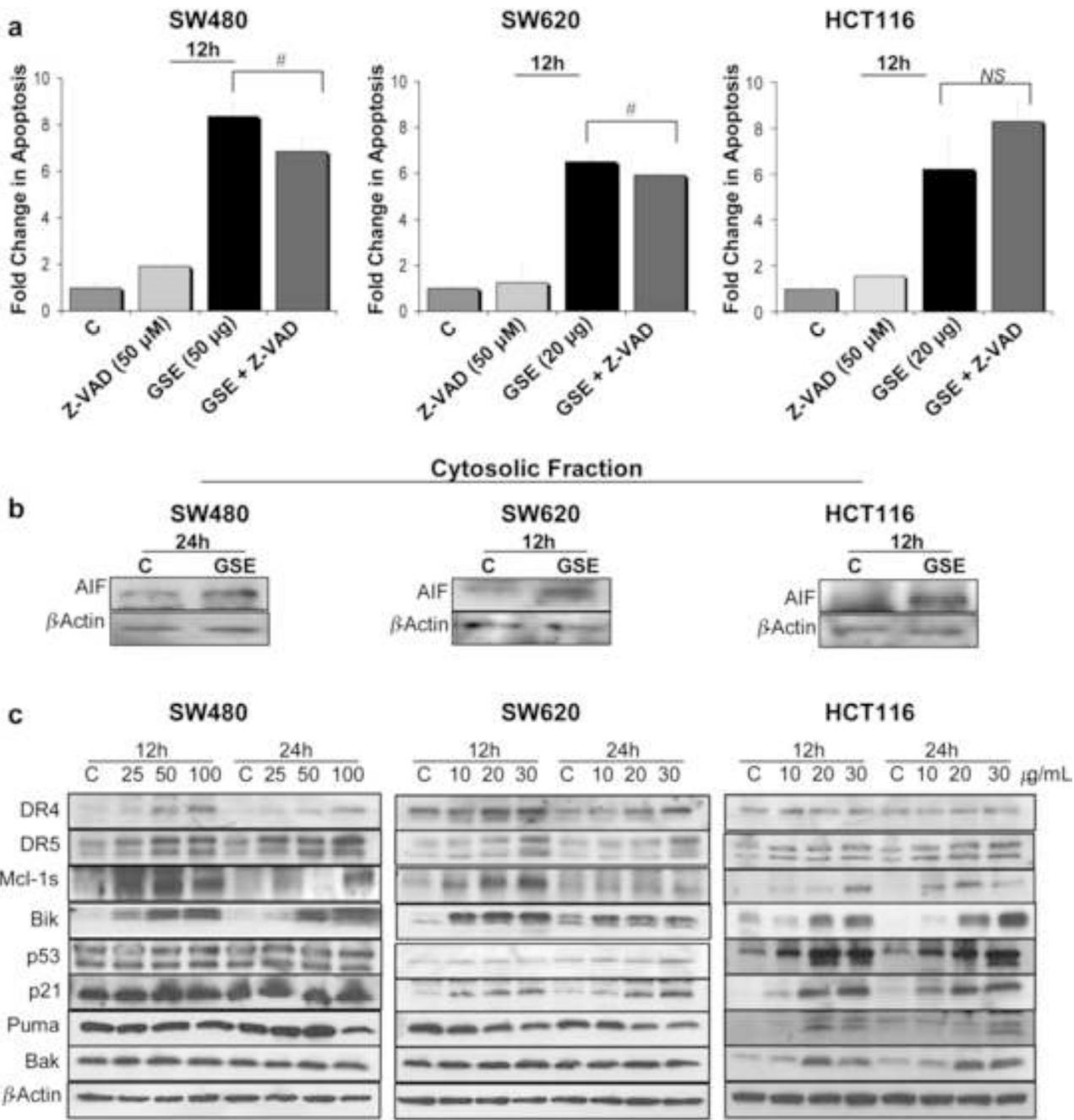


Figure 4. GSE treatment causes caspase-dependent and caspase independent apoptotic cell death in CRC cells

A) Effect of pre-treatment (2 h) with all-caspase inhibitor Z-VAD-Fmk (50 μM) on GSE (20–50 μg/mL, 12 h) induced apoptotic death in CRC cells. % apoptotic death of CRC cell is represented by columns (mean values of three independent samples for each treatment ± S.E.M). # P 0.01. **B)** Apoptotic effect of GSE is accompanied with the release of AIF in the cytoplasm of CRC cells. Membranes were also stripped and re-probed with anti-GADPH antibody to confirm equal protein loading. **C)** Apoptotic effect of GSE involves differential

up regulation of Death receptors and modulation of the expression of pro- and anti-apoptotic proteins. S.E.M, standard error mean.

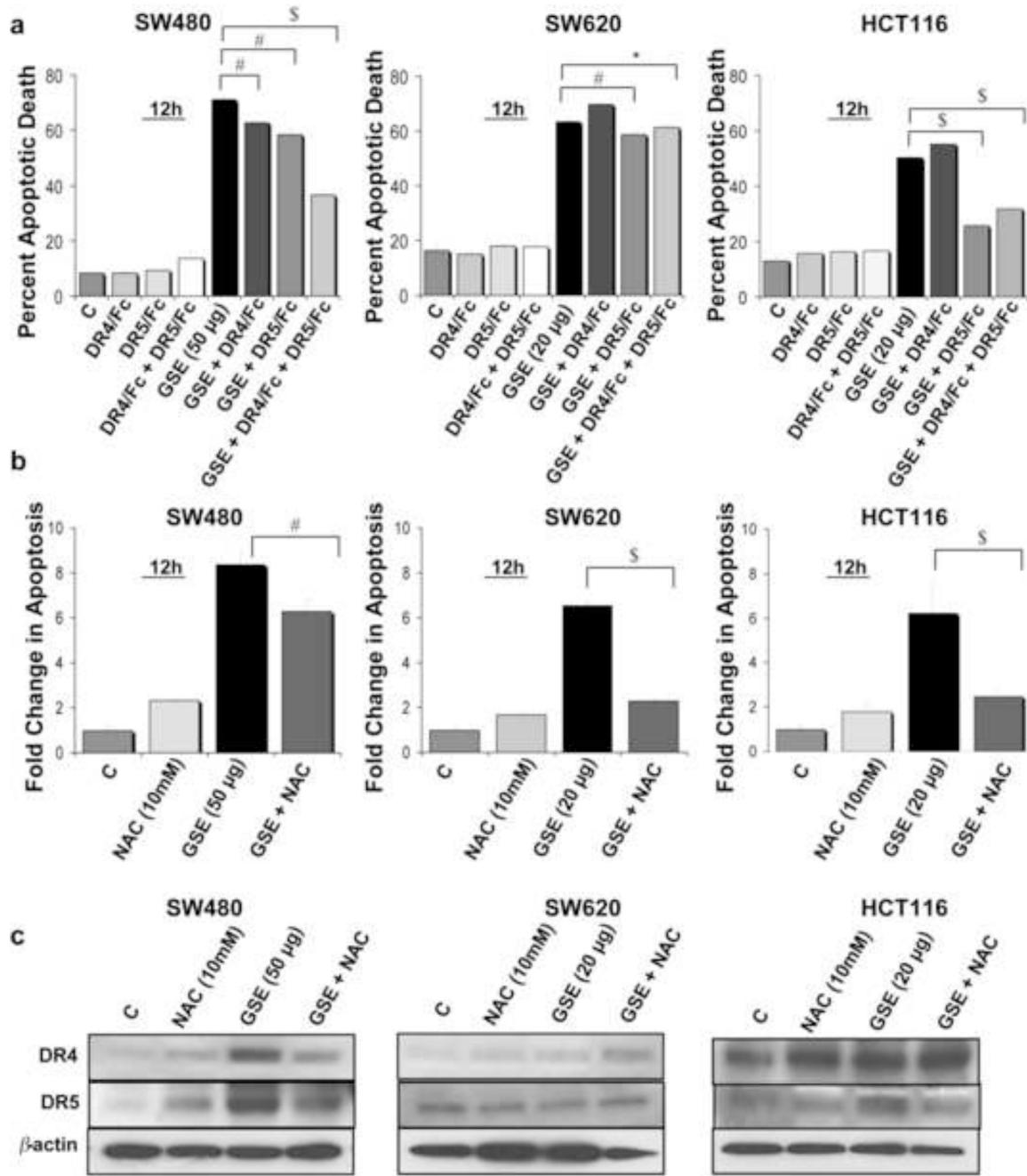


Figure 5. GSE-mediated apoptotic death involves oxidative stress mediated up regulation of Death receptors DR4 and DR5

A) Effect of inhibition of Death receptor mediated signaling on the apoptotic effect induced by GSE in CRC cells. CRC cells were either pre-treated for 2hr. with DR4/Fc (100ng/mL) or DR5/Fc (100ng/mL) chimeric proteins or a combination of both, prior to GSE exposure (20-50µg/mL, for 12h) and then % apoptotic death was evaluated. Effect of pre-treatment of CRC cells with anti-oxidant, 10mM NAC, for 1hr prior to GSE exposure on, B) Apoptotic effect induced by GSE, C) modulation of Death receptors by GSE. * P 0.05; #P 0.01; \$ P 0.001. S.E.M, standard error mean.

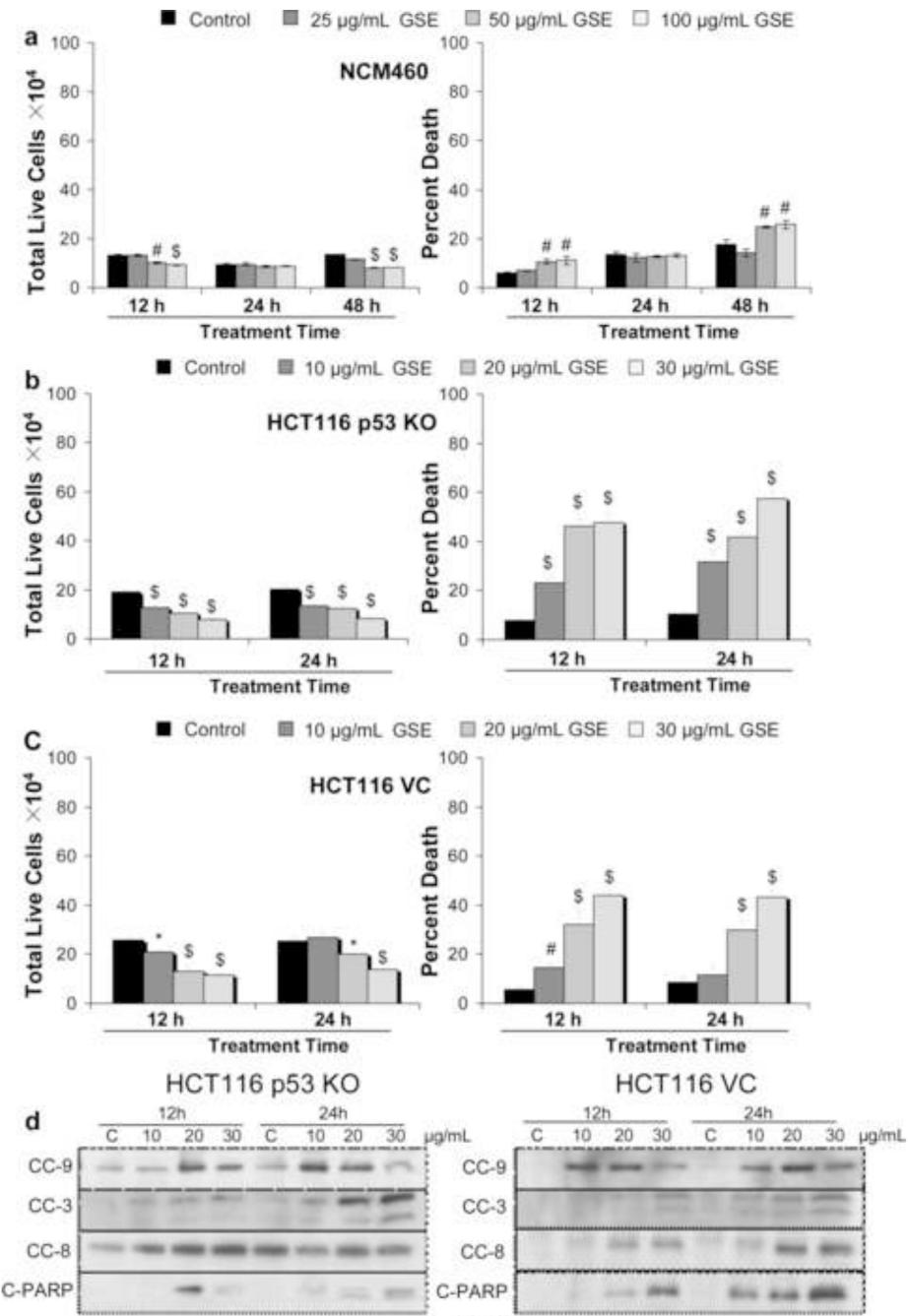


Figure 6. GSE-mediated cellular death is independent of p53 status of human CRC cells
The cytotoxic effect of Kikkoman GSE on, A) normal colon epithelial cells, NCM460; B) HCT116 p53 KO, and C) vector control, HCT116 VC cells. Effect on cellular proliferation and viability of cells was measured by assessing total live cell number and percent dead population after GSE treatment GSE doses ranged from 10-100µg/mL, and effect was observed for 12-48 hrs. The data shown here is representative of the mean of 3 independent values; bars, S.E.M; * P 0.05; # P 0.01; \$ P 0.001; control (DMSO). D) Effect of GSE

exposure on cleavage of caspases-9, -8, -3 and PARP in HCT116 p53 KO and HCT116 VC cells. S.E.M, standard error mean.