Diallyl disulfide, from garlic oil, synthesizes human colonic adenocarcinoma cell line (Caco-2) to TNF-alpha-mediated apoptosis through up-regulation of membrane FAS levels

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Abstract: Colon cancer cells resistance to apoptosis inducing factors, particularly those working through the extrinsic apoptotic rout, such as Tumour necrosis factor alpha (TNF-α), may due to that these cells lack of the presence of FAS receptor on their membrane, to which TNF-α binds and causes the recruitment of caspase-8 and other caspases cascade required in apoptotic cell death. In addition to its reported impact as anti-tumour dietary elements, Diallyl disulfide (DADS), a major organosulfur compound found in garlic oil, was examined in this research for its ability to induce membrane FAS expression in colonic adenocarcinoma cell line (Caco-2), and thereby synthetizes such cells to TNF-alpha-mediated apoptosis. Our results show that the administration of DADS to Caco-2 cell cultures significantly increased the number of dead cells, exhibited morphological changes characteristic to apoptosis and induced caspas-3 cleavage but not caspase-8. Western blot along with flow-cytometry data confirmed the induction of FAS expression, at the protein level, after DADS treatment, RT-PCR also confirmed FAS expression, at the transcriptional level. This resulted in restoring Caco-2 cells sensitivity to TNF-α and initiating the extrinsic pathway through FAS and caspase-8 cleavage. These results suggest that DADS induces apoptosis in colonic cancerous cells not only through the intrinsic pathways but also through enhancing the extrinsic pathway.

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1. Introduction

Membrane FAS receptor or tumour necrosis factor receptor superfamily member 6 (TNFRSf6), is expressed in a variety of cell types including tissues largely characterized by high rates of cellular turnover and apoptotic cell death, such as colonic epithelial cells [1]. FAS receptor functions as cell surface-antigen when binds to FAS ligand forms the death-inducing signalling complex (DISC), this results in cleavage of caspase-8 and initiation of apoptotic cell death [2, 3]. The sensitivity of FAS mediated apoptosis in a given cell type correlates with the level of FAS expression on the cell membrane. However, FAS receptors are highly expressed in normal human colonic epithelial cells, they are steadily decreased or commonly lost during cancer progression, making many types of cancerous cells less- or completely insensitive to FAS mediated apoptosis [4-6]. Tumour necrosis factor alpha (TNF-α), a cytokine produced primarily by macrophages, is known to induce apoptosis through binding to membrane FAS receptors [7]. However, many cell types are resistant to TNF-α death receptor mediated killing. Caco-2 (human colonic adenocarcinoma cell line) is among such cell types that has no detected FAS receptors on their membrane surface [4, 8]. Which is the reason of making such kind of colonic adenocarcinoma cells resistant to apoptosis inducing factors that work through FAS rout, *e.g.*, tumour necrosis factors family (TNF-α and FAS ligand [FAS-L]) [9, 10]. This has led to the hypothesis of that the induction of FAS expression on the surface of Caco-2 cells may restore the sensitivity of such resistant cancer cells to FAS dependent apoptosis inducing factors [11, 12].

The impact of certain dietary components in colon cancer progression or prevention is having lots of scientific debate nowadays. Epidemiological studies conducted by collaborations of nutritionists, cell and molecular biologists have categorized certain dietary components into colon cancer inducing dietary agents, *e.g.*, alcohol and red meat [13], higher salted foods [14], high fat diet [15], food colouring extracts from some plants such as *Rubia tinctorum* [16], food additives such as *Aloe arborescens* Mill var extracted from *Aloe* plant a member of the family *Liliaceae* [17], and on the other hand colon cancer protective dietary

agents, *e.g.*, green tea extract [18, 19], apple polysaccharides [20], fermented milk products [21], leafy vegetables rich in folic acid and fibres [22-24], tomato enriched with lycopene [25], tributyrin, a short-chain triglyceride oil [26], olive oil [27], seafoods and soya foods [28, 29], fruits rich in polyphenolic compounds, delphinidin and kaempferol, a major component of flavonols [30-32], and konjac glucomannan [33]; However the mechanism of action of such dietary components remains unclear. In addition to traditional use as a spice, garlic (*Allium sativum*) has well known medicinal benefits [34]. Diallyl disulfide (DADS), a major organosulfur compound found in garlic oil is reported to exhibit various pharmacological properties such as antimicrobial [35], hypolipidemic [36], antithrombotic [37], and anti-carcinogenic [38]. However, little is known about the mechanism by which such an active compound works. Some reports showed that DADS plays a role in P53 and cyclin B1 protein regulation, induces cell cycle arrest, inhibits matrix metalloproteinase and thereby stops migration and invasion of cancerous cells and induces histone acetylation [39-42]. Other reports indicated that DADS induces a significant rise in Ca^{2+} in colon cancer cells by stimulating both extracellular Ca^{2+} influx and intracellular Ca^{2+} release [43]; this suggests that DADS indirectly modulate apoptosis in colon cancer cells as Ca^{2+} level is known to affect Bcl-2 family proteins, proteolytic enzymes of apoptosis, ensuing alterations on the signalling patterns of extracellular stimuli and the intracellular targets of apoptotic Ca^{2+} signals, with special emphasis on the mitochondria and cytosolic Ca^{2+}-dependent enzymes [44].

The impact of DADS on up-regulation of some genes involved in cellular apoptosis of colonic cancer cells was also studied. Genes such as tumour suppressor p53, pro-apoptotic Bcl2 protein (Bax) and cell cycle regulator cyclin B were up-regulated in HT29 and COLO 205 colonic cancer cell lines upon DADS treatment [45, 46]. In this study we investigated the role of DADS in increasing the susceptibility of colonic cancer cells (Caco-2) to apoptosis induced by TNF-α. Our results suggested the ability of DADS to synthetize human colonic adenocarcinoma cell line (Caco-2) to TNF-α dependent apoptosis through up-regulating membrane FAS expression at both levels RNA and protein.

2.Materials and Methods

Cell culture and treatments

Caco-2 cells [47] were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, CAMBREX) supplemented with 25 mM glucose, 20% inactivated (30 min, 56 °C) foetal calf serum (Sigma), penicillin (50 U/ml), streptomycin (50 U/ml) and 1% nonessential amino acids (Sigma). The cell line was used between passages 163 and 181. Cells were seeded in a concentration of 2 x 10^4 cells/cm^2 in 25 cm^2 tissue culture flasks (Corning), and after 24 h cultures were either supplied with DADS (Sigma) (final concentration of 200 µM) for another 24 h or left without any supplements. TNF-α (Sigma) was administrated to both cultures (final concentration of 15 ng/ml) in order to obtain four different groups of cultures as the following: none treated or control cultures (have neither DADS nor TNF-α), DADS treated cultures, TNF-α treated cultures and cultures supplemented with TNF-α after 24 h of DADS administration.

Cell viability

Viable cell counts were conducted using Trypan Blue method and haemocytometer according to Freshney, [48]. Briefly, 0.1 ml of 0.4% Trypan Blue Stain (Sigma) was added to 0.5 ml of a cell suspension diluted in complete medium without serum to a concentration of 2 x 10^5 cells per ml and mixed thoroughly. The cells were allowed to stand for 5 min at room temperature, and then cells (viable non-stained and dead blue stained) were counted using haemocytometer.

Nucleic acid stain (DAPI staining)

DAPI staining was performed using DAPI dihydrochloride nucleic acid stain (Invetrogen). Cells were harvested; both detached cells (collected by centrifugation) and attached cells (dissociated by 0.01% trypsin-EDTA [Cambrex]) combined together to obtain cell suspension. Cells were pelleted by centrifugation at 200 x*g* for 5 min and re-suspended in 1 ml PBS at room temperature. Cells were then fixed by pipetting the cell suspension over 4 ml of absolute ethanol at -20 ˚C while vortexing at top speed and left for 15 min at -20 ˚C. After centrifugation and discarding ethanol, fixed cells were rehydrated by adding 5 ml PBS and kept for 15 min at room temperature and pelleted again by centrifugation, 200 x*g* for 5 min. DAPI [1 ml of 3µM in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet P-40)] was added and cells were incubated for 15 min at room temperature. Stained cells were then centrifuged, 200 x*g* for 5 min, and the pellet was re-suspended in 0.5 ml of fresh staining buffer and one drop was applied into a microscope slide and covered with a coverslip for microscopic analysis. Images were acquired through AXIOCAM and AXIOVISION software and Carl Zeiss fluorescent microscope.

Western Blotting

Total protein was extracted from 10^7 Caco-2 cells by adding 1ml of Radio-Immuno Precipitation Assay buffer [RIPA (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml PMSF [phenylmethylsulphonyl fluoride], 1 µg/ml aprotinin, 1% NP-40)]. Cells were incubated for 30 min

on crushed ice. The cellular lysate was then centrifuged at 12,000×*g* for 2 min at 4 °C in order to eliminate the cellular debris. The supernatant was then transferred to a fresh Eppendorf tube, and total protein concentrations were determined using the BioRad protein assay [49]. Denaturation of protein samples was performed by adding loading buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8), 1:1 (v/v) at 100 °C for 10 min followed by centrifugation 3000×*g* for 1 min. Samples (50 µg of protein) were immediately electrophoresed by SDS-PAGE (BioRad Mini Protein II Electrophoresis gel). Molecular weight markers (See Blue® Plus 2 pre-stained protein standards, UK) were used as a size ladder. Protein bands were transferred to a nitrocellulose membrane (Amersham). The membranes were then incubated in blocking solution [5% non-fat dry milk in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5)] overnight on a shaker at 4 °C, immuno-stained for pro- and active caspases -8 and -3 using rabbit polyclonal primary antibodies (Abcam), and for membrane FAS receptor using mouse anti-FAS monoclonal antibody (Stressgen), and for β-Actin using anti- β-Actin mouse monoclonal primary antibody (Abcam) as a loading control, was conducting according to manufacturer's instructions. Immuno-detected band visualisation was carried out using the chemiluminescent alkaline phosphatase substrate (Immobilom™ Western).

Flowcytometric analysis of membrane FAS expression:

Membrane FAS quantification by flowcytometry was carried out according to [50]. Harvested cells were re-suspended in serum-free tissue culture medium containing 4 µg/ml mouse anti-FAS monoclonal antibody (Stressgen) and incubated at 4 °C for 45 min. Cells were washed twice in serum-free mediul and re-suspended again in serum-free medium containing FITC-conjugated goat anti-mouse IgG, x200 diluted, and incubated for an additional 30 min at 4 °C. Cells were then washed twice with PBS. The expression of membrane FAS receptors was assessed using a flow cytometer (Becton Dickinson). Data analysis was carried out using WinMDI 2.8 and Microsoft Excel software.

RNA purification and RT-PCR of FAS mRNA

Caco-2 cells were cultured as above, the culturing medium was supplemented with DADS (200 µM), 5-aza-2-deoxycytidine [Sigma-Aldrich] (5-azadC, 1 µM), in order to induce membrane FAS expression in Caco-2 cells [4], or none. After 24 h Cells were harvested and total RNA was isolated using TRIZOL® Reagent (Invitrogen) according to manufacture instructions. RNA (2µg) was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacture instructions. Amplification of cDNA for FAS and β-actin were conducted using semi-quantification PCR and specific primer pairs [4].

FAS specific primer pair:

[F] 5′- GACCCAGAATACCAAGTGCAGATGTA
[R] 5′-CTGTTTCAGGATTTAAGGTTGGAGATT

β-actin specific primer pair:

[F] 5′-GTGGGGCGCCCCAGGCACCA
[R] 5′-CTCCTTAATGTCACGCACGATTTC

The PCR conditions for amplification of FAS and β-actin cDNA to yield 289 and 540 bp products respectively were: initial denaturation at 94˚, 3 min; 25 cycles at 94˚, 1 min; 60˚, 2 min; 72˚, 2 min; and a final extension of 72˚, 4 min. The PCR products were resolved on 1.2% agarose gel, stained with Ethidium bromide and visualized by UV.

Statistical analysis

All data were obtained from 3 independent experiments, and all results are expressed as mean ±SE. We employed the 2-tailed (or paired) Student's t-test, using Microsoft Excel, to determine significant differences. In all analysis, differences with probability values ≤ 0.05 were considered significant.

3. Results

Apoptosis induction by DADS and/or TNF-α

Trypan blue staining showed significant increase in dead cells, 1.53 fold ($p \leq 0.0013$), upon DADS treatment (200 µM) compared with the dead cells numbers in the control cultures, while there was no significant change observed after TNF-α (15 ng/ml) treated cells ($p \leq 0.18$). However, dead cells in cultures supplemented with DADS for 24 h prior to TNF-α administration for another 24 h were significantly greater in number, 2.34 fold ($p \leq$ 8.86E-09), than it in case of control cultures, and also was significantly greater, 1.54 fold ($p \leq$ 1.01E-05), than the dead cells numbers in cultures treated with DADS alone (Fig. 1A).

Nucleic acids staining (DAPI) reviled morphological changes characteristics to apoptosis, nuclear shrinkage and fragmentation (Fig. 1B), in the cells treated with DADS alone, for 48 hrs, and also in the cells treated with DADS, for 24 hrs followed by TNF-α for another 24 hrs. such morphological changes were not seen neither in control cells or in cells treated with TNF-α alone for 48 hrs.

Caspase-3 and -8 activation

Caspases activation was monitored by Western blotting of cellular extracts of Caco-2 cells using specific antibodies for caspase-3 or caspase-8. The active forms (cleaved) of caspase-3 were seen as protein bands at 17 and 21 kDa in cells treated with DADS as well as in cells treated with DADS, for 24 hrs followed by TNF-α for another 24 hrs, indicative of caspase-3 activation; and no active caspase-3 bands were seen in the control cells and in the cells treated

with TNF-α alone (Fig. 2A). Cleaved caspase-8 bands, at (18/20) kDa were seen only in cells treated with DADS for 24 hrs followed by TNF-α for another 24 hrs. none of active caspase-8 bands were seen in control, DADS and TNF-α treated cells (Fig. 2A).

Induction of membrane FAS expression

The above data showed that DADS treatment (200 μM) 24 hrs prior to TNF-α administration to Caco-2 cells (15 ng) for another 24 hrs, initiated an additional apoptotic cell death pathway, which is caspase-8 route. As active caspase-8 bands were not seen in all other treatments. Caspase-8 activation upon TNF-α treatment is a downstream molecular event of TNF-α – membrane FAS binding [2]; however, Caco-2 cells are among colonic cancer adenocarcinoma cells that have no FAS receptors expressed on their membrane [4, 8]. This suggested DADS may induce FAS expression in Caco-2 cells. In order to confirm this hypothesis membrane FAS expression was examined at both protein and mRNA levels.

Western Blot analysis of Caco-2 cellular lysates using specific anti-FAS antibody showed protein band specific to FAS at 48 kDa in cultures treated with DADS and no such protein band was seen in control or TNF-α treated cultures (Fig. 2A). Concurrently, mean channel fluorescence (MCF) acquired by flocytometeric analysis of Caco-2 cells labelled with specific anti-FAS primary antibody and FITC-conjugated secondary antibody, showed that MCF values of Caco-2 cells increased up to ~3 fold ($p \leq$ 1.85E-09) greater than those of control or TNF-α treated Caco-2 cells (Fig. 2B).

In order to determine the impact of DADS at the transcriptional level, total RNA was isolated and cloned into cDNA. Levels of FAS mRNA ware monitored in Caco-2 cells using simi-quantitative PCR and specific primer pairs for FAS transcript. 5-azadC is a chemical compound known to induce membrane FAS expression in Caco-2 cells [4], so that it was used as positive control. Analysis of PCR products on agarose gel electrophoresis showed clear DNA bands at 298 bp specific to FAS transcript in Caco-2 cells treated with 1 μM 5-azadC as well as in cells treated with 200 μM both for 48 h (Fig. 3A). Gel analysis for band volume was performed using TotalLab^{TM} software based on reference band volume of DNA ladder (HyperLadder I, Bioline, UK) entered to the software. The obtained data determined that FAS band volume in case of DADS treatment was 18.63 ng, which is ~2 fold less (37.27 ng) than it in case of 5-azadC treated cells and no detected bands in control cells; while there were no significant differences among band volume values of transcribed β-actin mRNA in control, 5-azadC or DADS treated cells (Fig. 3B).

4. Discussion

DADS synthetizes Caco-2 cells to TNF-α induced apoptosis

FAS receptors are not expressed on Caco-2 cell Membrane [8] making such colonic adenocarcinoma cells resistant to FAS-mediated death; the hypothesis of restoring the sensitivity of Caco-2 cells to FAS-mediated apoptosis, through induction of membrane FAS expression, has been previously proven by Petak *et al*., [4]; they induced the expression of FAS using 5-azadC, and as a result Caco-2 cells responded to the extrinsic apoptotic signal induced by anti-FAS antibody (CH-11). In this research we studied one of the most popular dietary ingredient, garlic oil, particularly Diallyl disulphide (DADS), a major organosulfur compound found in garlic oil in a quantity of more than 60% [51], for its ability to synthetizes Caco-2 cells to TNF-α (the cytokine naturally produced by macrophages) -mediated apoptosis through up-regulation of membrane FAS levels. The results presented here showed that Caco-2 cells are insensitive to TNF-α and there was no significant difference between numbers of the dead cells in control and TNF-α treated cultures; and no morphological changes characteristic to apoptosis were seen. However, numbers of the dead cells, in DADS treated cells were significantly greater than those of control, 1.53 fold ($p \leq$ 0.0013), with clear morphological changes, such as apoptotic bodies and nuclear shrinkage, indicative of apoptosis. DADS induced apoptosis in Caco-2 cells were previously investigated, Jakubikova *et al*., reported that the apoptotic effect of DADS implies in its ability to induces G_2/M cell cycle arrest [38], Druesne *et al*., stated that DADS could inhibit nuclear histone deacetylase activity [52], other studies by Ban *et al*., and Yang *et al*., indicated that DADS induces apoptosis in colon cancer cell lines through the induction of reactive oxygen species, endoplasmic reticulum stress, caspases cascade and mitochondrial-dependent pathways [53, 54]. The results currently presented in this study are consistent with the above mentioned regarding DADS ability to elicit intrinsic apoptotic pathway through caspase-3 activation but not capase-8 (Fig. 2). However, active caspase-8 bands were detected in the cellular lysate of Caco-2 cells treated with TNF-α after 24 hrs of DADS administration. Recruitment of active caspase-8 is involved in the apoptotic cell death induced by membrane FAS upon binding to FAS ligand or TNF-α cytokine. This causes the recruitment of FAS-Associated protein Death Domain (FADD) followed by activation of caspase-8 [3]. The detection of active caspas-8 in cells treated with DADS prior to TNF- α, but not in cells treated separately with DADS or TNF- α, led to the suggestion that FAS expression on Caco-2 cell membrane may occur as a result of DADS treatment; and in order to

examine this possibility, FAS expression was monitored at both protein and transcriptional levels.

DADS induces FAS receptor expression on Caco-2 cell membrane

The obtained results confirmed the induction of FAS expression. At the protein level, Western blotting of Caco-2 cellular lysate, using anti-FAS monoclonal antibody specific for FAS, detects FAS protein band at 48 kDa in cells treated with DADS but not in control or cells treated with TNF- α alone. Similarly, flocytometric analysis, FITC conjugated secondary antibody, quantified the difference between the florescence emitted from control and DADS treated cells population by ~3 fold greater ($p \leq$ 1.85E-09) (Fig. 2). At the transcriptional level, FAS mRNA was detected in positive control samples, cells treated with 5-azadC [4], and in DADS treated cells, but not in control or TNF-α treated cells (Fig. 3). However, the impact of 5-azadC was ~2 fold greater than it of DADS. These results confirm that DADS induced FAS receptor expression on Caco-2 cell membrane at both levels, transcriptional and protein expression, which explains caspase-8 cleavage as a result of TNF-α binding to FAS receptor, and also explains the maximisation of apoptotic signal (working through intrinsic as well as extrinsic pathways) seen in the high number of dead cells in cultures treated with DADS 24 h prior to TNF-α treatment, 1.54 fold ($p \leq$ 1.01E-05) greater than the dead cells numbers in cultures treated with DADS alone, and 2.34 fold ($p \leq$ 8.86E-09) greater than the dead cells numbers in control cultures.

The above findings re-introduce DADS as a multipotent anticancer dietary element induces apoptosis in colonic cancerous cells not only through the intrinsic pathways, as previously proved, but also through enhancing the extrinsic pathway, as we proved, mediated by the induction of membrane FAS expression and recruiting the initiator caspas-8 cleavage.

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Abbreviations

DISC	death-inducing signalling complex
FAS-L	FAS ligand
TNF-α	Tumour necrosis factor alpha
5-azadC	5-aza-2-deoxycytidine
DADS	Diallyl disulfide
DAPI	4',6-diamidino-2-phenylindole
FITC	Fluorescein isothiocyanate
PBS	Phosphate Buffered Saline
RIPA	Radio-Immuno Precipitation Assay buffer

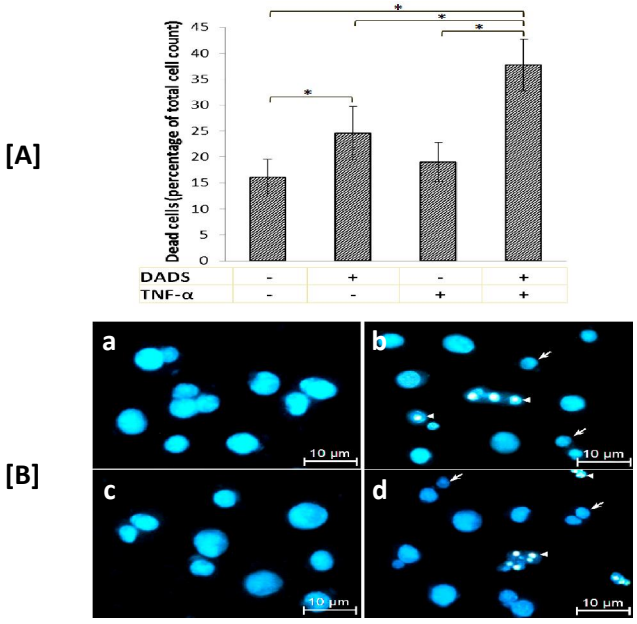

Figure 1. [A]; Dead cells count via Trypan blue dye exclusion method (expressed as percentage of total cell count) in Caco-2 cell cultures in response to DADS and / or TNF-α treatment. The results are reported as mean ± SE of 3 independent experiments. ∗Significant ($P \leq 0.05$). **[B]**; Fluorescence photomicrograph, x 100 objective (bar = 10 µm), of Caco-2 cells stained with DAPI nucleic acid binding dye; **(a)** none treated control, **(b)** DADS treated, **(c)** TNF-α treated and **(d)** cells treated with TNF-α after 24 h of DADS administration as in materials and methods. Arrows show nuclear condensation and arrow heads show fragmented nuclei and apoptotic bodies.

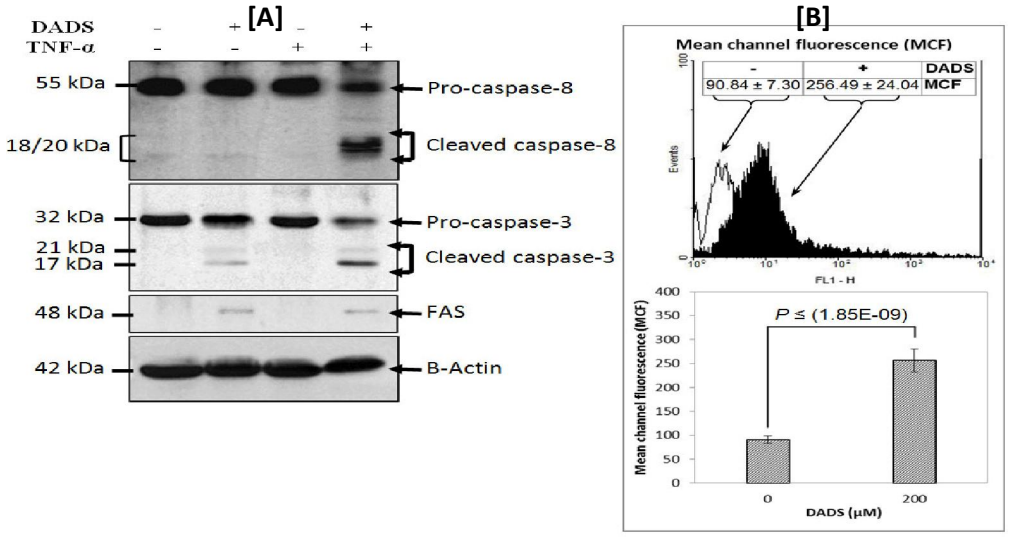

Figure 2. [A] Western blot analysis of different cellular lysates of Caco-2 cells, none treated control, treated with DADS and / or TNF-α as in materials and methods. The blots show the impact of such treatments on caspase-8 and -3 cleavages and on the expression of membrane FAS receptors; β-actin antibody was used as a loading control. **[B]** Flowcytometric histogram shows the induction of membrane FAS expression in Caco-2 cells after DADS treatment. Cells were labelled with anti-FAS monoclonal antibody, followed by FITC-conjugated secondary antibody. Mean channel fluorescence (MCF) was acquired by flowcytometer (Becton Dickinson) and data were analysed using WinMDI 2.8 software. Data are expressed as mean of three independent experiments; ($P \leq 0.05$) considered significant.

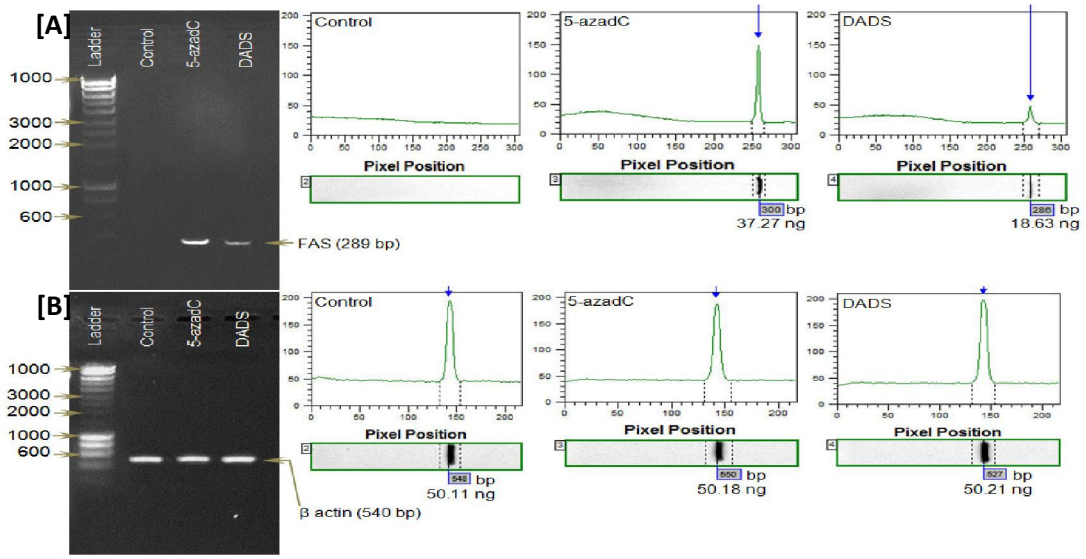

Figure 3. Agarose gel electrophoresis of PCR product and gel analysis using TotalLab^{TM} software, show the up-regulation of FAS expression by 5-azadC and DADS **[A];** and β-actin (loading control) **[B]** in Caco-2 cell line. Total RNA was reverse transcribed into cDNA; amplification of cDNA of FAS and β-actin mRNA were conducted using semi-quantification PCR and specific primer pairs as in materials and methods. Agarose gel and software analysis show that FAS mRNA was up-regulated (289 pb) in both 5-azadC and DADS treated cells and no bands were detected in control cells; however the pixel intensity and band volume were greater in 5-azadC than in DADS treated cells. No such an effect is seen in β-actin bands (540 pb), as both pixel intensity and band volume were uniform (to large extent) in control and treated cells.

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