available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

5-Aminosalicylic acid enhances anchorage-independent colorectal cancer cell death

Daniele Fina[a,e], *Luigi Franchi*[b,e], *Roberta Caruso*[a], *Ilaria Peluso*[a], *Gian Carlo Naccari*[c], *Salvatore Bellinvia*[c], *Roberto Testi*[d], *Francesco Pallone*[a], *Giovanni Monteleone*[a,*]

[a]*Dipartimento di Medicina Interna, Cattedra di Gastroenterologia, e Centro di Eccellenza per lo studio delle malattie complesse e multifattoriali, Università Tor Vergata, Via Montpellier, 1, 00133 Rome, Italy*
[b]*University of Michigan Medical School, Department of Pathology and Comprehensive Cancer Center, Ann Arbor, Michigan 48109, USA*
[c]*Giuliani Spa, Milan, Italy*
[d]*Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università Tor Vergata, Rome, Italy*

ARTICLE INFO

Article history:
Received 11 January 2006
Received in revised form
7 March 2006
Accepted 10 March 2006
Available online 17 August 2006

Keywords:
Anoikis
5-ASA
Colon cancer

ABSTRACT

Resistance to anoikis, the cell death triggered by the loss of anchorage to the substratum, is an essential prerequisite in the proliferation and diffusion of colorectal cancer (CRC) cells. We examined whether 5-aminosalicylic acid (5-ASA), a drug that seems to reduce the risk of colitis-associated CRC, enhances CRC cell anoikis. To this end, Colo205 cells were treated with 5-ASA in the presence or absence of inhibitors of caspases (zVAD-fmk) and reactive oxygen species (ROS). We demonstrate that 5-ASA enhances Colo205 cell death. Although 5-ASA induces dissipation of mitochondrial transmembrane potential and caspase-3 activation, zVAD-fmk does not completely prevent the 5-ASA-induced cell death. 5-ASA also enhances the synthesis of ROS. However, inhibitors of ROS reduce the fraction of 5-ASA-induced Colo205 cell death but do not confer protection. In contrast, the 5-ASA-mediated Colo205 cell death is preventable by Bcl-2 over-expression. These data suggest a mechanism by which 5-ASA interferes with colon carcinogenesis.

1. Introduction

Anoikis, a form of programmed cell death triggered by the loss of anchorage to the extracellular matrix, contributes to maintain tissue homeostasis, particularly in the gut, where epithelial cells continuously undergo death and shedding into the lumen.[1] Resistance to anoikis is a common property of many tumours of epithelial origins, and it is supposed to play a major role in the growth and diffusion of carcinomas.[2] Indeed, it is well known that solid tumours grow *in vivo* as multicellular masses in which at least a proportion of cells is deprived of normal contacts with the basement membrane and is anoikis-resistant.[2] Additionally, most cell lines derived from such solid tumours are capable of growing in an anchorage-independent manner as colonies in soft agar or suspension culture.[3] In line with these findings, suppression of the resistance against anoikis in transformed epithelial cells strongly inhibits their tumourigenicity.[4,5] The precise molecular mechanism(s) by which cancer cells usurp normal extracellular matrix-derived survival signals and acquire resistance to anoikis are not fully characterised. However, recent studies have shown that activation of some intracellular pathways contributes to enhance the survival of cancer cells. In particular, epidermal growth factor receptor (EGFR), nuclear factor-kB, and

* *Corresponding author:* Tel.: +39 06 72596158; fax: +39 06 72596391.
E-mail address: Gi.Monteleone@Med.uniroma2.it (G. Monteleone).
[e] These authors contributed equally to this study.
0959-8049/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2006.03.030

the non-receptor protein tyrosine kinase, Src, have been implicated in the resistance of cancer cells to anoikis.[1,5]

Despite considerable advance in our understanding of colorectal cancer (CRC) pathogenesis, the overall incidence of this neoplasia is yet increasing, and CRC represents the second most common fatal malignancy in Western world.[6] This is largely due to the lack of effective treatment of advanced disease. Evidence has been accumulated to show that non-steroidal anti-inflammatory drugs (NSAIDs) are effective in preventing/limiting both the development and progression of CRC in the general population.[7,8] However, the frequent and often severe side effects occurring in subjects taking NSAIDs have limited the use of these drugs in CRC therapy.[9,10]

More recently, epidemiological studies have suggested that regular intake of the clinically well-characterised 5-aminosalicylic acid (5-ASA), the drug of choice in the maintenance of remission and treatment of mild flare-ups of inflammatory bowel disease (IBD), reduces the risk of CRC developing in patients with ulcerative colitis.[11,12] It is plausible that the 5-ASA-related anti-neoplastic effect relies on the ability of this drug to inhibit specific inflammatory pathways that contribute to the development of CRC complicating IBD. However, studies both *in vitro* and animal models of sporadic CRC have shown that 5-ASA may have direct and negative effects on the growth of CRC cells.[13–15] We have also recently shown that 5-ASA disrupts EGFR signalling both in primary and CRC cell lines,[16] further confirming the anti-neoplastic effect of this drug. The documented effect of 5-ASA on EGFR-associated signal pathway prompted us to explore the possibility that this drug could increase the susceptibility of non-adherent CRC cells to death. To this end, we first assessed the *in vitro* effect of 5-ASA on Colo205, a CRC cell line that is able to spontaneously grow in suspension. Second, we extended our analysis to HT-29 in suspension culture, given that this CRC cell line has been reported to be highly resistant to the anchorage-independent death.[17] The molecular mechanism by which 5-ASA triggers cell death was also investigated.

2. Materials and methods

2.1. Cell lines and culture conditions

The two human CRC cell lines, HT-29 and Colo205, were cultured in appropriate media in absence of antibiotics in a humidified incubator under 5% CO_2 at 37 °C.

Colo205 cells over-expressing Bcl-2 were selected after the transfection of pcDNA3-Bcl-2 by electroporation (Nucleofector, Amaxa GmbH, Koeln, Germany). To generate stably transfected cell lines, Colo205 cells were maintained in culture in the presence of 500 μg/mL neomycin (G418, Inalco S.p.A., Milan, Italy). The empty pcDNA3 vector was used to produce a neomycin-resistant pool for control experiments.

5-ASA (Giuliani S.p.A., Milan, Italy) was dissolved as a 100 mM stock solution in culture medium just before using and used at concentrations of 10–50 mM. The pH of the drug solution was adjusted to 7.4 with NaOH, and experiments were carried out protected from light. Mannitol (50 mM, pH 7.4; Sigma–Aldrich, Milan, Italy) was used as both an osmolar and pH control, and staurosporine (500 ng/mL; Sigma–Aldrich) was used as a positive control of cell death.

Colo205 (2×10^5/well) were cultured in RPMI 1640 containing 5% fetal bovine serum (FBS) and plated on a 12-well dish, with or without 5-ASA, mannitol or staurosporine for 2–24 h. In parallel, cells were cultured in the presence of a pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fmk (zVAD-fmk; 20–100 μM; DBA Italia, Milan, Italy) and/or GSH (0.2–5 mM, Sigma–Aldrich) for 1 h prior to adding 5-ASA. HT-29 cells (2×10^5/well) were cultured in McCoy medium containing 5% FBS, in the presence or absence of 5-ASA or mannitol, plated on a 12-well dish and maintained in suspension by gentle agitation for 6–12 h. To examine the effect of 5-ASA on adherent cells, HT-29 cells were cultured in 12-well plates, and after reaching the confluence were either left untreated or treated with graded doses of 5-ASA, staurosporine or mannitol for 6–12 h.

2.2. Analysis and quantification of cell death

To score cell death, cells were incubated with 5 μg/ml propidium iodide (PI; Sigma–Aldrich) and stained with FITC-annexin V (AV; 1:100 final dilution, Becton Dickinson, Milan, Italy) for 20 min at 4 °C, and their fluorescence was measured using FL-1 and FL-2 channels of FACSCalibur flow cytometer (Becton Dickinson) using Cell Quest Pro software.

2.3. Analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$) and reactive oxygen species (ROS) production

To analyse whether 5-ASA alters the $\Delta\Psi_m$, CRC cells were either left untreated or treated with 50 mM 5-ASA for the indicated time points. Cells were incubated with 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] (20 μM, Invitrogen S.r.L., Milan, Italy) during the last 15 min of culture. Production of ROS was assessed in CRC cells treated with 5-ASA (50 mM) or acetyl salicylic acid (ASA, 10 mM, Sigma–Aldrich) for 2–8 h. Cells were incubated with dihydroethidium (DHE, 5 μM, Invitrogen S.r.L) or 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA, 5 μM, Sigma–Aldrich) during the last 20 min of culture, and then evaluated by flow cytometry.

2.4. Western blotting analysis

Cytosolic proteins were separated on a gradient SDS–PAGE gel, and then incubated with a polyclonal rabbit anti-human caspase-9 (1:500 final dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or monoclonal anti-human caspase-3 antibody (2 μg/ml; Vinci-Biochem, Florence, Italy) or monoclonal anti-human poly-ADP-ribosepolymerase-1 (PARP-1) antibody (1:1000 final dilution; Vinci-Biochem). Additionally, cytosolic extracts were prepared from cells either left untreated or treated with 5-ASA for 4–12 h and used for analysis of Bax, Bak, Bcl-2, AIF, cytochrome c and Omi/HtrA2 by Western blotting. To this end, we used commercially available antibodies (Bax, Bak, Bcl-2, and AIF, 1:500 final dilution, from Santa Cruz Biotechnology, while cytochrome c and Omi/HtrA2, 1:500 final dilution, were purchased from Becton Dickinson and Analitica De Mori S.r.l. Milan, Italy, respectively). Binding of the primary antibody was detected using a horseradish peroxidase-conjugated secondary antibody (Dako S.p.A., Milan, Italy) (final dilution

1:20,000) and chemiluminescent substrate (Pierce, Rockford, IL, USA). At the end, each blot was stripped and incubated with a mouse-anti-human monoclonal β-actin antibody (1:5,000; Sigma–Aldrich) to ascertain equivalent loading of the lanes.

2.5. *Caspase-3 activity assay*

Caspase-3 activity was measured using a tetrapeptide *p*-nitroanilide (pNA) substrate in a colorimetric assay. The assays were performed in 96-well plates by incubating 40 μg of proteins in a final volume of 100 μl reaction buffer (100 mM HEPES, pH 7.5, 20% glycerol, 5 mM DTT, and 0.5 mM EDTA) containing 100 μM of N-Acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA; Sigma–Aldrich). Absorbance at 405 nm was monitored at 37 °C for 1 h using a microplate reader (Bio-Rad Laboratories, Milan, Italy) and values expressed as arbitrary units (a.u.).

2.6. *Statistical analysis*

Values are expressed as means ± SD. To evaluate the difference in means between groups, the Student *t* test was used and significance was defined as *p* values less than 0.05.

3. Results

3.1. *5-ASA induces death of colon cancer cells growing in suspension*

To assess the effect of 5-ASA on non-adherent CRC cells, we first treated Colo205 cells with graded doses of 5-ASA. Exposure of Colo205 to 25 and 50 but not 10 mM 5-ASA resulted in enhanced cell death (Fig. 1a, $p < 0.01$). This effect was evident as early as 4 h after 5-ASA exposure and increased over the time course (Fig. 1b, $p < 0.01$). Importantly, 5-ASA increased the percentage of both AV-positive/PI-negative cells (35 ± 4% and 22 ± 5% in cells treated with 50 and 25 mM 5-ASA respectively versus 8 ± 1% in untreated cells; $p < 0.01$) and AV-positive and PI-positive cells (15 ± 7% and 12 ± 2.5% in cells treated with 50 and 25 mM 5-ASA respectively versus 3 ± 1.5% in untreated cells, $p < 0.02$). In contrast, an increase in AV-negative/PI-positive cells was seen in cultures added with 50 mM (25 ± 5%) but not 25 mM (11 ± 2.5%) 5-ASA compared to untreated cultures (8 ± 2%) ($p < 0.02$).

To further confirm these results we then used a well-characterised method in which HT-29 cells were removed from their substrate and kept in suspension with gentle agitation. HT-29 cells were selected given that previous studies have shown that these cells survive when kept in suspension.[17] 5-ASA significantly increased the percentage of death in non-adherent HT-29 cells. Time-course analysis revealed that after 6 h treatment with 50 mM 5-ASA, 26 ± 5% of cells were AV positive, but PI negative, versus 6 ± 4% in untreated cells ($p = 0.03$), whereas the percentage of AV positive/PI positive cells was respectively 19 ± 12% in 5-ASA-treated cells versus 13 ± 10% in untreated cells (p = NS). After 12 h culture with 5-ASA, many of the cells were positive for both AV and PI (AV: 13 ± 5%; AV/PI: 53 ± 10% versus 11 ± 3% and 12 ± 6% respectively in untreated cells) indicating that they had completed apoptosis and were now necrotic. No significant change in cell death was seen when cells were treated with equivalent concentrations of mannitol, indicating that the effect of 5-ASA on HT-29 cell anoikis was not secondary to osmotic shifts in the culture medium. Importantly, the percentage of death in adherent HT-29 cells after 12 h culture was increased by staurosporine (31 ± 5%) but not 50 mM 5-ASA (9 ± 3% versus 8 ± 4% in untreated cells) clearly indicating that 5-ASA only reduces the survival of HT-29 cells in suspension culture.

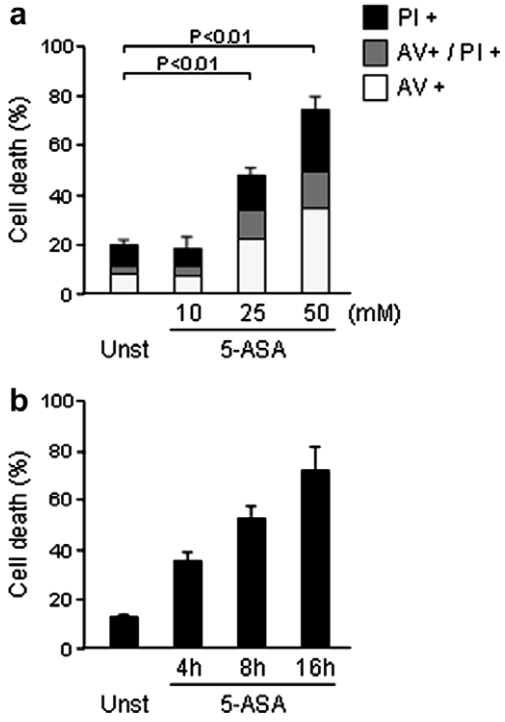

Fig. 1 – (a) 5-ASA significantly enhances Colo205 cell death at concentrations of 25 and 50 mM ($p < 0.01$). Colo205 were either left unstimulated (Unst) or treated with the indicated concentrations of 5-ASA for 16 h. Data indicate the mean percentages of cell death as assessed by FACS analysis of AV and PI-positive cells. Vertical bars indicate the SD of six separate experiments and refer to the total cell death. (b) 5-ASA significantly enhances Colo205 cell death in a time-dependent fashion ($p < 0.01$). Colo205 were either left unstimulated (Unst) or treated with 50 mM 5-ASA for the indicated time points. Data are expressed as mean ± SD of five separate experiments and indicate the percentage of total cell death as assessed by FACS analysis of AV and PI-positive cells.

3.2. *5-ASA induces loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) in CRC cells*

Mitochondria play a critical role in the regulation of cell death in response to a wide variety of stimuli, including anti-cancer drugs.[18] Therefore we evaluated the dissipation of $\Delta\Psi_m$ in cells treated with 5-ASA. Reduction of $\Delta\Psi_m$ was evident as early as 2 h after 50 mM 5-ASA exposure and increased over the time course. However, differences between untreated and 5-ASA-treated cells became significant only after 4 h of treatment (Fig. 2a, $p < 0.01$). To examine whether the 5-ASA-

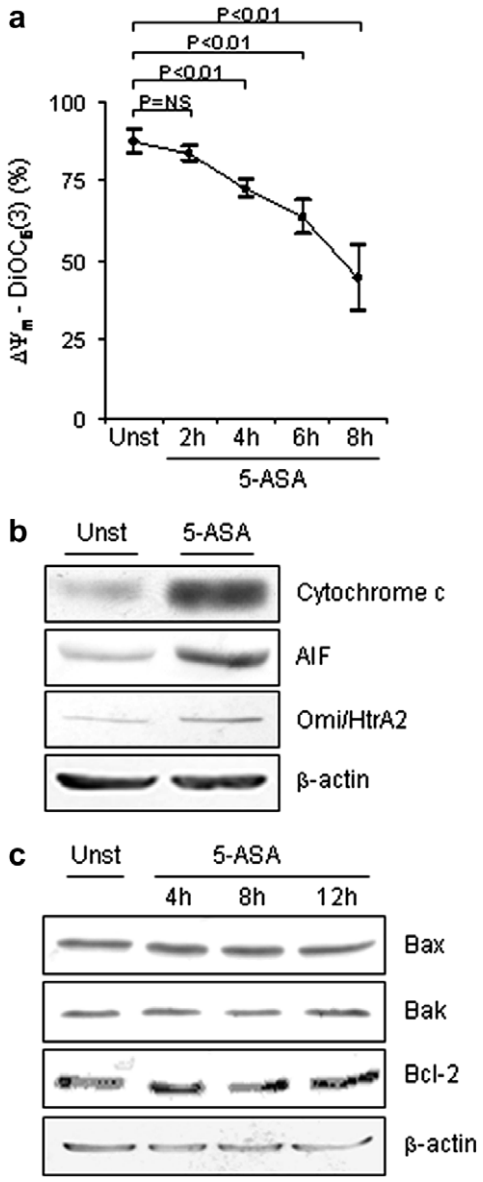

Fig. 2 – (a) 5-ASA induces a time-dependent reduction of $\Delta\Psi_m$ ($p < 0.01$). Colo205 cells were either left unstimulated (Unst) or treated with 50 mM 5-ASA for the indicated time points, then incubated with $DiOC_6(3)$, followed by analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$). Data are expressed as mean ± SD of five separate experiments and indicate the percentage of cells with intact $\Delta\Psi_m$ as assessed by flow cytometry. (b) Treatment of Colo205 cells with 5-ASA enhances the cytosolic accumulation of mitochondrial proteins. Cytosolic extracts were prepared from cells either left untreated (Unst) or treated with 50 mM 5-ASA for 8 h and then analysed for the indicated proteins by Western blotting. One of three representative experiments is shown. (c) 5-ASA does not alter the expression of Bax, Bak and Bcl-2 in Colo205 cells. Cytosolic extracts were prepared from Colo205 cells either left untimulated (Unst) or treated with 50 mM 5-ASA for 4, 8 and 12 h, and analysed for the indicated proteins by Western blotting. One of four representative experiments is shown.

induced reduction in $\Delta\Psi_m$ was reversible, cells were either left untreated or treated with 50 mM 5-ASA for 6–8 h, then extensively washed and cultured in fresh drug-free medium for a further 12 h. At the end, $\Delta\Psi_m$ was evaluated by flow cytometry. In two representative experiments, the percentages of cells with intact $\Delta\Psi_m$ were 89 ± 2% in cells pre-cultured with medium and 75 ± 4 and 67 ± 5% in cell pre-treated with 5-ASA for 6 and 8 h respectively, suggesting that loss of $\Delta\Psi_m$ induced by 5-ASA is not fully reversible after removing the 5-ASA from the culture medium. As mitochondrial depolarisation is accompanied by the release of proteins, including cytochrome c, AIF and Omi/HtrA2, into the cytoplasm,[18] we examined whether 5-ASA enhanced the intra-cytoplasmic content of these molecules. To this end, cytosolic extracts, prepared from Colo205 cells either left untreated or treated with 5-ASA for 8 h, were analysed for the content of cytochrome c, AIF and Omi/HtrA2 by Western blotting. As shown in Fig. 2b, 5-ASA enhanced the accumulation of all these mitochondrial proteins into the cytoplasm.

As $\Delta\Psi_m$ loss may be in part regulated by influencing the expression of pro- and anti-apoptotic members of the Bcl-2 family,[19] we also examined whether 5-ASA changed the cytosolic level of such proteins. Neither Bax nor Bak expression was affected by 5-ASA. Similarly, 5-ASA did not alter the content of the anti-apoptotic protein Bcl-2 and this was evident at each time point analysed (Fig. 2c).

3.3. *The ability of 5-ASA to enhance anchorage-independent CRC cell death is not entirely preventable by caspases inhibitors*

In response to apoptotic stimuli, mitochondrial proteins released into the cytoplasm activate APAF-1 that in turn recruits and activates capase-9, an initiator caspase.[18] To examine whether the 5-ASA-induced loss of $\Delta\Psi_m$ was followed by caspase-9 activation, cytosolic proteins, prepared from Colo205 cells either left untreated or treated with 5-ASA (50 mM), were analysed for caspase-9 by Western blotting. In parallel, cells were treated with staurosporine as a positive control of caspase activation. Data in Fig. 3a (upper blot) show that stimulation of cells with 5-ASA caused a time-dependent reduction of the full-length form of caspase-9, even though no cleaved band was detectable. Similar findings were observed when cells were treated with staurosporine. Caspase-3 is an executioner caspase that triggers a number of key cell death-specific events, and it has been reported to be activated in intestinal epithelial cells undergoing apoptosis.[20] Therefore we evaluated whether 5-ASA activated caspase-3. To this end, Colo205 cells were treated with 50 mM 5-ASA for different time points and caspase-3 activation monitored by Western blotting by using a specific monoclonal antibody that recognises both the full-length and cleaved forms of this enzyme. 5-ASA induced a time-dependent reduction in the intensity of the 32 kDa band corresponding to the full-length form of caspase-3, and this was paralleled by the appearance of the cleaved band of such an enzyme over the time course (Fig. 3a, middle blot). Similar data were obtained when 5-ASA was added to HT-29 cells kept in suspension culture (not shown). Analysis of caspase-3 activity by using a colourimetric substrate confirmed the inducing effect of 5-ASA on

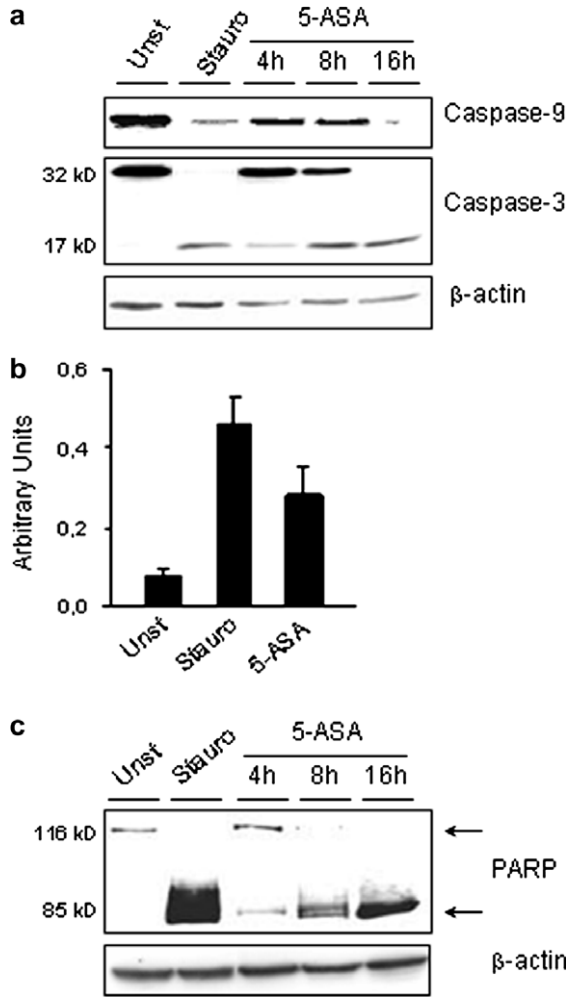

Fig. 3 – (a) 5-ASA triggers caspase activation in CRC cells. Representative Western blots showing both caspase-9 (upper blot) and caspase-3 (middle blot) in proteins prepared from Colo205 cells either left unstimulated (Unst) or treated with staurosporine (Stauro) for 16 h or 50 mM 5-ASA for 4, 8 and 16 h. After analysis of caspases, the blot was stripped and incubated with an anti-human β-actin antibody. One of six representative experiments is shown. (b) 5-ASA enhances caspase-3 activity in Colo205 cells. Colo205 cells were either left unstimulated (Unst) or treated with staurosporine or 50 mM 5-ASA for 8 h, followed by caspase-3 activity assay. Data are expressed in densitometric arbitrary units (a.u.) and indicate mean ± SD of three separate experiments. (c) Effect of 5-ASA on PARP-1 activation. Representative Western blot showing both full-length and cleaved forms of PARP-1 in protein extracts of Colo205 cells cultured as indicated in (a). After analysis of PARP-1, the blot was stripped and incubated with an anti-human β-actin antibody. One of four representative experiments is shown.

the activation of this enzyme (Fig. 3b). As activated caspase-3 cleaves PARP-1 from its 116 kDa to an 85 kDa residual fragment, we confirmed the activation of caspase-3 through Western blotting for PARP-1 cleavage products. As expected, treatment of Colo205 with 5-ASA resulted both in a time-dependent reduction in the intensity of the 116 KDa band and induction of the 85 KDa cleaved form of PARP-1 (Fig. 3c). Similar results were seen in HT-29 cells growing in suspension while neither caspase-3 nor PARP-1 was affected by 5-ASA in adherent HT-29 cells (not shown).

To examine the involvement of caspases in the 5-ASA-induced lethal effects, Colo205 cells were pre-incubated with zVAD-fmk or DMSO and subsequently treated with 5-ASA or staurosporine. Importantly, in zVAD-fmk-treated Colo205 cells, the activity of caspase-3 was constantly maintained below the control level (Fig. 4a), thus confirming that this inhibitor was active in our system. No inhibitor effect was seen when cells were pre-incubated with DMSO (not shown). Flow cytometry analysis revealed that pre-incubation of Colo205 cells with zVAD-fmk significantly reduced the percentage of AV-positive/PI-negative cells induced by 5-ASA (15 ± 2% versus 34.7 ± 4%, $p = 0.02$), whereas the fractions of AV-positive/PI-positive cells (18 ± 9 versus 21.5 ± 13) and AV-negative/PI-positive (4 ± 3 versus 2.5 ±2.7) were not significantly modified (Fig. 4b). This effect was observed at all the applied doses of zVAD-fmk (not shown). These data suggest that 5-ASA enhances Colo205 cell death through a mechanism which is only in part dependent on caspase activity.

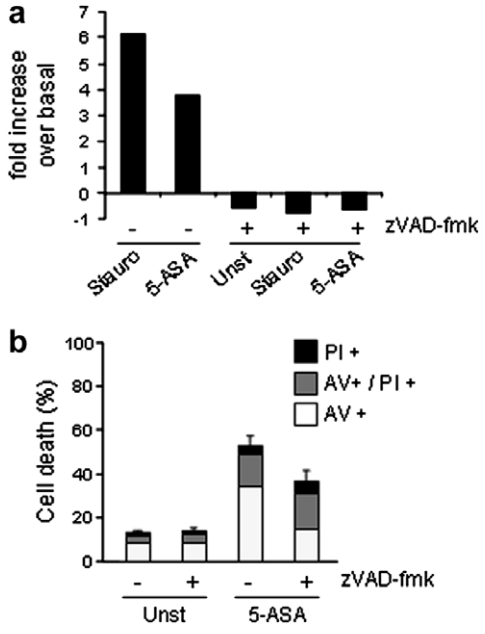

Fig. 4 – The pancaspase inhibitor, zVAD-fmk, partially inhibits the 5-ASA-induced Colo205 cell death. (a) Colo205 cells were preincubated with zVAD-fmk or DMSO (vehicle) for 1 h prior to adding staurosporine (500 ng/ml) or 5-ASA (50 mM) for an additional 8 h. Proteins were then analysed for caspase-3 activity. Data indicate fold increase as compared with control (DMSO-treated cells) and express mean of three separate experiments. (b) Colo205 cells were preincubated with medium (Unst) or 100 μM zVAD-fmk for 1 h and then treated or not with 50 mM 5-ASA. After 16 h cells were recovered and analysed for AV and PI positivity. Data indicate the mean percentages of AV, PI and AV/PI-positive cells of four separate experiments, while vertical bars indicate the SD and refer to the total cell death. zVAD-fmk significantly reduces the percentage of AV- ($p = 0.02$) but not the fraction of PI-positive cells induced by 5-ASA.

3.4. 5-ASA enhances the production of peroxides in CRC cells

Since ROS are important mediators of caspase-independent cell death,[21] we then evaluated the production of ROS by staining with DHE and H_2-DCF-DA, which are oxidised in the presence of superoxide and peroxides, respectively, to fluorescent dyes. ASA but not 5-ASA increased the production of superoxide in Colo205 (Fig. 5a, $p < 0.01$). In contrast, both ASA and 5-ASA were effective in enhancing the production of peroxides (Fig. 5b, $p < 0.01$ and $p = 0.03$, respectively).

To examine the role of peroxides in the 5-ASA-mediated anoikis, Colo205 cells were pre-incubated with reduced glutathione (GSH), an antioxidant molecule, and then treated with 5-ASA. We first showed that GSH dose-dependently inhibited the 5-ASA-induced peroxides production (Fig. 5c). Since 5 mM GSH completely suppressed the 5-ASA-induced peroxides production, we then examined the effect of such a dose of GSH on the 5-ASA-induced Colo205 cell death. Pre-incubation of cells with GSH significantly reduced the percentage of 5-ASA-induced AV-positive and PI-positive cells (8 ± 2.5% versus 22 ± 14% in cells treated with 5-ASA alone, $p = 0.03$) but not the fraction of AV-positive/PI-negative cells (19 ± 16% versus 20 ± 14% in cells treated with 5-ASA alone) (Fig. 5d). Overall, GSH significantly reduced the percentages of cell death induced by 5-ASA (Fig. 5d, $p < 0.001$). Additionally, the percentage of 5-ASA-induced cell death was decreased further by pre-treatment of cells with both zVAD-fmk and GSH in comparison to GSH alone, even if the use of both inhibitors did not confer full protection (Fig. 5d). Similar results were observed in non-adherent HT-29 cells (not shown).

3.5. Over-expression of Bcl-2 prevents the 5-ASA-induced CRC cell anoikis

These data raise the possibility that 5-ASA promotes mitochondrial damage and activation of multiple cell death pathways. Therefore, we investigated whether protection of mitochondria by over-expression of Bcl-2 could prevent the 5-ASA-induced cell death. To this end, Colo205 cells were stably transfected with either a pc3/Bcl-2 or pc3 (control) plasmid, and then treated with 5-ASA. Importantly, cells transfected with pc3/Bcl-2 but not empty vector contain elevated amount

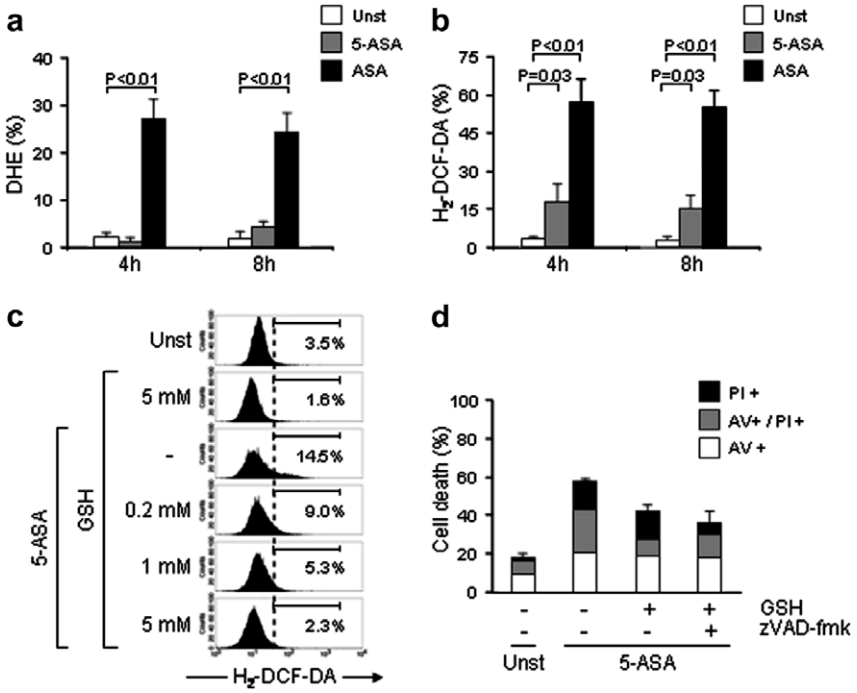

Fig. 5 – GSH, an inhibitor of peroxide production, reduces but does not abolish the 5-ASA-induced Colo205 cell death. (a) Colo205 cells were either left unstimulated (Unst) or treated with 50 mM 5-ASA or ASA (10 mM) for the indicated time points, followed by measurement of the intracellular production of superoxide and peroxides by DHE (a) and H2-DCF-DA (b) staining respectively. Data are expressed as mean ± SD of three separate experiments. ASA significantly enhances the production of both superoxide and peroxides ($p < 0.01$), whereas 5-ASA significantly enhances only the production of peroxides ($p = 0.03$). (c) GSH dose-dependently inhibits the 5-ASA-induced peroxides synthesis. Colo205 cells were pre-incubated with graded doses of GSH for 1 h followed by 5-ASA for 8 h. Peroxides production was analysed as indicated above. One of three separate experiments is shown. (d) Effect of GSH on 5-ASA-induced Colo205 cell death. Cells were either left unstimulated (Unst) or pre-incubated with GSH (5 mM) in the presence or absence of zVAD-fmk (100 μM) for 1 h followed by 5-ASA for a further 8 h. Data are expressed as mean ± SD of three separate experiments and indicate the percentage of cell death as assessed by analysis of AV and/or PI-positive cells. GSH significantly inhibits the percentages of AV+/PI+ cells ($p = 0.03$) and total cell death ($p < 0.001$) but not the fraction of apoptotic cells (AV+/PI− cells) induced by 5-ASA. The combination of GSH and zVAD-fmk further reduces the 5-ASA-induced total cell death ($p < 0.001$) but does not confer full protection.

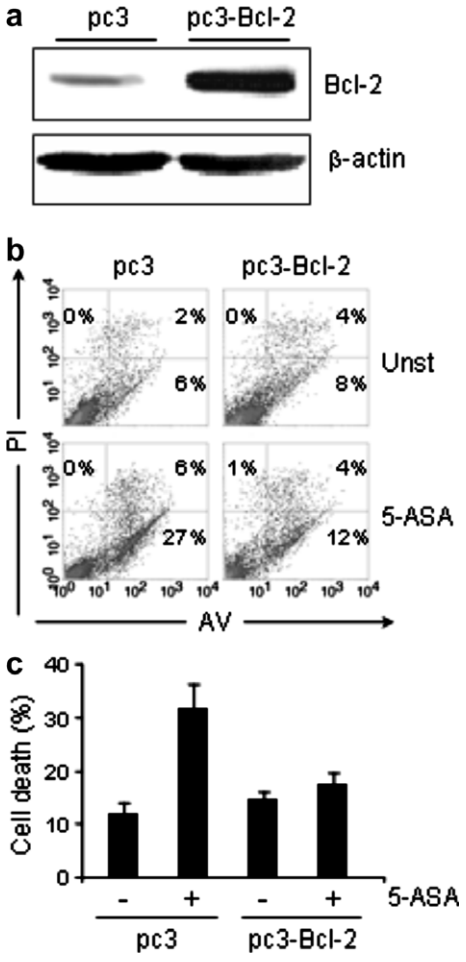

Fig. 6 – Stable over-expression of Bcl-2 prevents the 5-ASA-induced Colo205 cell death. (a) Representative Western blot showing Bcl-2 and β-actin in protein extracts of Colo205 cells stably transfected with either empty pcDNA3 vector (pc3) or pcDNA3-Bcl-2 plasmid (pc3-Bcl-2). (b) Colo205 cells stably transfected with empty pcDNA3 vector (pc3) or pcDNA3-Bcl-2 plasmid (pc3-Bcl-2) were cultured in the absence (Unst) or presence of 50 mM 5-ASA for 8 h, and then analysed for AV and PI positivity. (c) Cells were cultured and analysed as indicated in (b). Data are expressed as mean ± SD of three separate experiments and indicate the percentage of cell death as assessed by analysis of AV and/or PI positive cells.

of Bcl-2 (Fig. 6a) and appeared resistant to the 5-ASA-induced death (Fig. 6b and c).

4. Discussion

Normal colonic cells require for their survival and growth signals elicited from adhesion to the extracellular matrix. Deprivation of anchorage destines cells to undergo apoptosis, a phenomenon termed anoikis.[1] In contrast, CRC cells survive when kept in suspension and this phenomenon is supposed to play a major role in the uncontrolled growth and metastasis of such tumours.[2] Attempts to either prevent or revert such an alteration are therefore worth pursuing for reducing the growth and/or diffusion of CRC cells.

This study was undertaken to investigate whether 5-ASA, a drug widely used in the management of patients with IBD and proposed as an attractive option for CRC chemoprevention[11,12] may enhance anchorage-independent CRC cell death. Using Colo205 and HT-29 cells as an *in vitro* model of CRC, we here show that 5-ASA enhances the death of non-adherent CRC cells. The 5-ASA effect on CRC cell death is seen at concentrations of drug which are higher than those reached within the gut tissue under standard oral treatment[22] but similar to those used by other authors to assess the anti-neoplastic effect of 5-ASA *in vitro*.[15] Our data also indicate that the 5-ASA-induced CRC cell death pathway converges on mitochondria. Indeed, treatment of CRC cells with 5-ASA is associated with a marked loss of $\Delta\Psi_m$, that occurs in the early phase of cell death and precedes the disruption of plasma membrane integrity. Moreover, we show that stable over-expression of Bcl-2 is sufficient to prevent the 5-ASA-induced lethal effect on CRC cells. The sequence of intracellular events that trigger the mitochondrial signalling pathway in 5-ASA-treated CRC cells remains, however, unclear. It is unlikely that 5-ASA uses the FAS signalling pathway to enhance CRC cell death, as 5-ASA does not promote caspase-8 activation, and incubation of CRC cells with graded doses of a neutralising FAS antibody prior to adding 5-ASA does not reduce the rate of 5-ASA-induced cell death (not shown). Moreover, analysis of pro- and anti-apoptotic members of the Bcl-2 family by Western blotting reveals no significant change in the global expression of Bax, Bak, and Bcl-2 after 5-ASA exposure.

Consistent with the $\Delta\Psi_m$ dissipation, 5-ASA-treated cells exhibit an accumulation of mitochondrial proteins into the cytoplasm and activation of caspase-3. However, pre-incubation of CRC cells with the broad caspase inhibitor, zVAD-fmk, reduces the percentage of 5-ASA-induced AV+/PI− cells but does not confer cytoprotection. Importantly, the effect of zVAD-fmk on the fraction of AV+/PI− cells is also maintained after long term 5-ASA exposure (i.e. 24 h) thereby indicating that zVAD-fmk really prevents the induction of apoptosis rather than delaying the manifestation of 5-ASA-mediated CRC cell death. Together, these data suggest that 5-ASA can trigger both caspase-dependent and -independent cell death signalling pathways. In this context, we show that 5-ASA increases the intracellular production of peroxides, and that suppression of peroxides production by GSH significantly reduces the percentage of 5-ASA-induced AV-positive and PI-positive cells. Moreover, the combination of GSH and zVAD-fmk further reduces but does not completely prevent the 5-ASA-induced cell death, raising the possibility that 5-ASA may activate multiple cell death signalling pathways. No reduction in the fraction of 5-ASA-induced CRC cell death was seen when Colo205 cells were treated with commercially available inhibitors of either non-caspase proteases or trypsin-like serine proteases (not shown), arguing against a role of these molecules in the 5-ASA-mediated cell death program.

Although the exact molecular basis underlying the ability of CRC cells to survive and grow under the non-adhesive or anchorage-independent conditions remains to be clarified, there is evidence that CRC cells constitutively activate several intracellular pathways that allow them to survive in suspension culture.[1,5] We have previously shown that treatment of HT-29 cells with 5-ASA results in a marked inhibition of EGFR,

a signalling pathway that is over-expressed in CRC cells and is supposed to inhibit apoptotic-inducing stimuli.[16,23,24] However, our unpublished observations would seem to suggest that the 5-ASA-induced CRC cell anoikis is not entirely dependent on the inhibition of EGFR activation. In fact, the percentage of Colo205 cell death induced by specific and powerful suppressors of EGFR activity was significantly lower than that induced by 5-ASA. Additionally, 5-ASA was effective in enhancing the death of EGFR-deficient neoplastic cells.

The balance between cell proliferation and apoptosis typically associated with the normal colonic epithelium becomes progressively dysregulated during the neoplastic transformation.[1] The fact that the *in vitro* property of CRC cells to survive and grow in suspension correlates very well with their *in vivo* oncogenic potential and that 5-ASA enhances anoikis of CRC cells further support the view that this drug may be a promising candidate for colitis-associated CRC therapy. In line with this, recent epidemiological studies have shown a favourable role of 5-ASA in the prevention of IBD-related CRC.[25,26] Clearly, an optimal study for ascertaining the chemopreventive effect of 5-ASA should be a prospective, randomised controlled study. However, given the long duration before dysplastic lesions or cancers develop, case-control studies and laboratory-based experiments could alternatively provide the best evidence to support the chemopreventive effect of 5-ASA in IBD patients.

Conflict of interest statement

None declared.

Acknowledgements

The study was supported by the Associazione Italiana per la Ricerca sul Cancro and Fondazione "Umberto Di Mario", Rome, Italy; L.F. was supported by a fellowship from Fondazione Italiana Ricerca sul Cancro.

REFERENCES

1. Grossmann J, Walther K, Artinger M, Kiessling S, Scholmerich J. Induction of apoptosis before shedding of human intestinal epithelial cells. *Am J Gastroenterol* 2002;**97**:1421–8.
2. Shanmugathasan M, Jothy S. Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int* 2000;**50**:273–9.
3. Freedman VH, Shin SI. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 1974;**3**:355–9.
4. Frankel A, Rosen K, Filmus J, Kerbel RS. Induction of anoikis and suppression of human ovarian tumor growth in vivo by down-regulation of Bcl-X(L). *Cancer Res* 2001;**61**:4837–41.
5. Grossmann J. Molecular mechanisms of "detachment-induced apoptosis–Anoikis. *Apoptosis* 2002;**7**:247–60.
6. Keighley MR. Gastrointestinal cancers in Europe. *Aliment Pharmacol Ther* 2003;**18**(Suppl 3):7–30.
7. Huls G, Koornstra JJ, Kleibeuker JH. Non-steroidal anti-inflammatory drugs and molecular carcinogenesis of colorectal carcinomas. *Lancet* 2003;**362**:230–2.
8. Smalley WE, DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. *Adv Pharmacol* 1997;**39**:1–20.
9. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *N Engl J Med* 2005;**352**:1071–80.
10. Silverstein FE, Faich G, Goldstein JL, et al. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib long-term arthritis safety study. *Jama* 2000;**284**:1247–55.
11. Eaden J. Review article: the data supporting a role for aminosalicylates in the chemoprevention of colorectal cancer in patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2003;**18**(Suppl 2):15–21.
12. Eaden J, Abrams K, Ekbom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment Pharmacol Ther* 2000;**14**:145–53.
13. Brown WA, Farmer KC, Skinner SA, Malcontenti-Wilson C, Misajon A, O'Brien PE. 5-aminosalicyclic acid and olsalazine inhibit tumor growth in a rodent model of colorectal cancer. *Dig Dis Sci* 2005;**45**:1578–84.
14. Narisawa T, Fukaura Y. Prevention by intrarectal 5-aminosalicylic acid of N-methylnitrosourea-induced colon cancer in F344 rats. *Dis Colon Rectum* 2003;**46**:900–3.
15. Reinacher-Schick A, Schoeneck A, Graeven U, Schwarte-Waldhoff I, Schmiegel W. Mesalazine causes a mitotic arrest and induces caspase-dependent apoptosis in colon carcinoma cells. *Carcinogenesis* 2003;**24**:443–51.
16. Monteleone G, Franchi L, Fina D, et al. Silencing of SH-PTP2 defines a crucial role in the inactivation of epidermal growth factor receptor by 5-aminosalicylic acid in colon cancer cells. *Cell Death Differ* 2006;**13**:202–11.
17. Windham TC, Parikh NU, Siwak DR, et al. Src activation regulates anoikis in human colon tumor cell lines. *Oncogene* 2002;**21**:7797–807.
18. LY JD, Grubb DR, Lawen A. The mitochondrial membrane potential ($\Delta\Psi_m$) in apoptosis; an update. *Apoptosis* 2003;**8**:115–28.
19. Daniel PT, Schulze-Osthoff K, Belka C, Guner D. Guardians of cell death: the Bcl-2 family proteins. *Essays Biochem* 2003;**39**:73–88.
20. Zhang HM, Rao JN, Guo X, et al. Akt kinase activation blocks apoptosis in intestinal epithelial cells by inhibiting caspase-3 after polyamine depletion. *J Biol Chem* 2004;**279**:22539–47.
21. Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 2002;**84**:131–41.
22. Frieri G, Giacomelli R, Pimpo M, et al. Mucosal 5-aminosalicylic acid concentration inversely correlates with severity of colonic inflammation in patients with ulcerative colitis. *Gut* 2000;**47**:410–4.
23. Resnick MB, Routhier J, Konkin T, Sabo E, Pricolo VE. Epidermal growth factor receptor, c-MET, beta-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. *Clin Cancer Res* 2004;**10**:3069–75.
24. Zhou Y, Brattain MG. Synergy of epidermal growth factor receptor kinase inhibitor AG1478 and ErbB2 kinase inhibitor AG879 in human colon carcinoma cells is associated with induction of apoptosis. *Cancer Res* 2005;**65**:5848–56.
25. Velayos FS, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005;**100**:1345–53.
26. van Staa TP, Card T, Leufkens HG, Logan RF. 5-Aminosalicylate use and colorectal cancer risk in inflammatory bowel disease: a large epidemiological study. *Gut* 2005;**54**:1573–8.