Polyunsaturated fatty acids augment tumoricidal action of 5-fluorouracil on gastric cancer cells by their action on vascular endothelial growth factor, tumor necrosis factor-α and lipid metabolism related factors

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Abstract

Introduction: Gastric cancer is the second most common cause of cancer-related mortality worldwide. 5-fluorouracil (5-FU) is a commonly used anti-cancer drug. Various polyunsaturated fatty acids (PUFAs) are known to have tumoricidal action both *in vitro* and *in vivo*. Though PUFAs are known to augment the cytotoxic action of anti-cancer drugs, the exact mechanism is not clear.

Material and methods: The human gastric cancer cell line MGC (undifferentiated) and human gastric cancer cell line SGC (semi-differentiated) were either 5-FU alone or a combination of 5-FU + PUFAs and their proliferation, and ability to secrete tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) and lipid metabolism-related factors lipoprotein lipase (LPL), peroxisome proliferator-activated-γ (PPAR-γ), and CCAAT enhancer-binding protein (C/EBP) were investigated and analyzed.

Results: It was noted that combined treatment of 5-FU + PUFAs on gastric carcinoma (MGC and SGC) cells produced a significant growth inhibitory action compared with either agent alone by inhibiting the production of TNF-α and VEGF and a simultaneous increase in the expression of LPL, PPAR-γ, and C/EBP.

Conclusions: Based on the results of the present study, it is concluded that PUFAs enhance the tumoricidal action of the anti-cancer drug 5-FU by acting on anti-angiogenic factors and enzymes involved in lipid metabolism.

Key words: gastric cancer, polyunsaturated fatty acids, nitric oxide, peroxisome proliferator-activated-γ, lipoprotein lipase.

Introduction

Gastric cancer is one of the most prevalent forms of cancer and is the second most common cause of cancer-related mortality worldwide [1, 2]. Despite its toxic side effects, 5-fluorouracil (5-FU), a pyrimidine analogue that interferes with thymidylate synthesis, is widely used in the treatment of gastric cancer and other neoplasms [3]. One of the major aims of cancer therapy is to selectively kill tumor cells with little or no effect on normal

cells. But this is rarely achieved with the current chemotherapeutic drugs. Hence, newer therapeutic approaches are needed that can either enhance the action of anti-cancer drugs such as 5-FU on tumor cells, reduce their side effects without compromising their anti-cancer action, or both. In this context, it is interesting to note that previously, we and others showed that certain polyunsaturated fatty acids (PUFAs) have selective tumoricidal action with little or no action on normal cells [4–10]. But, PUFAs themselves are not very effective in eliminating cancer, partly due to the fact that they are tightly bound to albumin and other proteins and hence are unavailable to tumor cells to bring about their tumoricidal action and may also be metabolized into several eicosanoids that may have other unwanted actions. Hence, it is desirable to develop methods whereby PUFAs are selectively delivered to tumor cells to produce their anti-cancer actions and/or given in combination with anti-cancer drugs so that the combined anti-cancer drug(s) + PUFAs may have a significant cytotoxic action on cancer cells compared to either agent alone. Previously, we and others observed that PUFAs and some of their products may also have anti-angiogenic action in addition to their ability to inhibit mitosis of tumor cells [11, 12]. Since PUFAs are lipid moieties, it is likely that they may have actions on enzymes or factors involved in lipid metabolism such as lipoprotein lipase (LPL), peroxisome proliferator-activated-γ (PPAR-γ) and CCAAT enhancer-binding protein (C/EBP). In the present study, we evaluated the combined effect of PUFAs + 5-FU on the growth and mechanism(s) involved in the induction of apoptosis of gastric carcinoma cells *in vitro*. Cancer is also a low-grade systemic inflammatory condition in which enhanced plasma levels of pro-inflammatory cytokines have been described [13, 14]. Similarly, vascular endothelial growth factor (VEGF), which is needed to enhance tumor angiogenesis, is also increased in the plasma of subjects with cancer [15–17].

In view of this, we assessed the effect of PUFAs and 5-FU on the secretion of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, and VEGF by gastric cancer cells *in vitro*.

Material and methods

α-Linolenic acid (ALA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), linoleic acid (LA), arachidonic acid (AA), γ-linolenic acid (GLA), 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), and 5-FU were purchased from Sigma (St. Louis, MO, USA). The human gastric cell lines MGC and SGC were kindly provided by Prof. P. Wensheng (Zhejiang University, China). RPMI medium 1640 was obtained from GIBCO (Grand Island, NY, USA). All other chemicals were of extra-pure grade or analytical grade.

Cell culture

MGC and SGC cells were maintained in RPMI-1640 medium, containing 10% fetal bovine serum and 100 U/ml penicillin-streptomycin at 37°C. The DHA, EPA, AA, GLA, ALA, and LA were dissolved in ethyl alcohol and 0.1 M NaOH respectively and filter-sterilized at a concentration of 10 mg/ml as a stock solution and diluted with sterile water before use. Fifty mM of 5-FU was dissolved in high purity water as a stock solution and was filtered prior to use.

MTT assay

MGC and SGC cells were seeded in 96-well plates at a density of 10,000 cells per well. After 24 h of seeding, cells were supplemented with mixed solution containing different concentrations of various PUFAs (ALA, DHA, EPA, LA, AA, and GLA) and 5-FU. Based on our previous studies, we selected the lowest dose of PUFAs (50 μM) and the highest dose (120 μM) that showed no cytotoxic action on normal cells [18] for the present study. The dose of 5-FU used ranged from 0.004 mM to 2 mM. Following 24, 48 and 72 h of treatment of MGC and SGC cells with various combinations of PUFAs + 5-FU, cell survival was assessed by MTT assay as described previously [18]. Both time-course and dose-response studies were performed with 5-FU and PUFAs.

Determination of VEGF and TNF-α level

For this study, MGC and SGC cells were seeded in 6-well culture plates at a density of 10,000 cells per well. After 48 h of addition of various mixtures of 5-FU + PUFAs, culture supernatant was collected, centrifuged at 2,000 × g for 5 min and the supernatant was collected for estimation of secreted VEGF and TNF-α using commercially available enzyme-linked immunosorbent assay (ELISA) kits purchased from Xitang (Shanghai, China) and BOSTER (Wuhan, China), respectively. The results obtained were represented as pg/mg protein.

Determination of PPAR-γ, C/EBP and LPL levels

MGC and SGC cells were treated with various mixtures of 5-FU + PUFAs as described above for 48 h. At the end of the incubation period of 48 h, the cell culture supernatant was collected, centrifuged and re-suspended in PBS and kept overnight at –20°C until analyzed. ELISA kits, obtained from CUSABIO, were used for the measurement of

PPAR-γ, C/EBP and LPL. The results obtained were represented as pg/mg protein.

Statistical analysis

Data obtained from various studies were expressed as mean ± SD. Data were analyzed with SAS 8.0 software. Significance of differences between groups was evaluated using the *t*-test.

Results

Effect of 5-FU on cell viability

Cells that were supplemented with different doses of 5-FU ranging from 0 to 2 mM for 24, 48 and 72 h showed decreased viability, as shown in Figure 1. Based on these results, we chose 0.004, 0.04, 0.1, 0.2, 0.4, 0.7 mM of 5-FU for the next set of studies in combination with the lowest and highest (50 µM and 120 µM) doses of PUFAs [18].

Effect of combination of 5-FU and PUFAs

At all the doses tested (Tables I and II), addition of PUFAs enhanced the inhibitory action of 5-FU on MGC and SGC cells. It is evident from these results that optimum combined concentrations of PUFAs and 5-FU were different for MGC and SGC cells, as shown in Tables I and II and Figure 1. As expected, the higher the dose of 5-FU, the higher the inhibitory effect of it on the proliferation of both MGC and SGC cells. When both MGC and SGC cells were exposed to a combination of 5-FU and higher and lower doses of various PUFAs, the inhibitory action of 5-FU on the proliferation of cells was enhanced. The effect of a combination of 5-FU and various PUFAs on the proliferation of MGC and SGC cells was as follows (taking into consideration both high and low concentrations of fatty acids): AA > EPA > DHA > ALA > GLA > LA on MGC cells; AA > ALA > EPA > DHA > GLA > LA on SGC cells (see Tables I and II). These results suggest that of all the fatty acids tested in combination with 5-FU, AA is the most effective in inhibiting the proliferation of both MGC and SGC cells *in vitro*. Based on these results, we used the most effective doses of 5-FU and various PUFAs. For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 µM, 50 µM, 50 µM, 120 µM, 50 µM and 120 µM respectively, and concentrations of 5-FU used were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, concentrations of ALA, DHA, EPA, LA, AA, and GLA were 120 µM, 50 µM, 50 µM, 50 µM, 120 µM and 120 µM respectively, and concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively in all subsequent studies.

Effect of 5-FU + PUFAs on VEGF and TNF-α secretion

Supplementation of MGC and SGC cells with 5-FU and PUFAs for 48 h produced a dramatic decrease in their ability to secrete VEGF and TNF-α, as shown in Figures 2 and 3.

Significant suppression of the secretion of TNF-α was noted by both SGC and MGC cells that were exposed to fatty acids in combination with 5-FU, except for EPA. Of all the fatty acids tested in combination with 5-FU, GLA showed the highest ability to suppress TNF-α secretion, whereas EPA showed the lowest inhibition. On a comparative basis, the effect of various fatty acids in combination with 5-FU on the secretion of TNF-α was as follows: GLA > DHA > AA > LA > ALA > EPA. On the other hand, the effect of various fatty acids in combination with 5-FU on SGC cells in their ability to suppress the production of TNF-α was as follows: GLA > LA > AA > ALA > EPA > DHA. γ-Linolenic acid (GLA) was the most potent in inhibiting the secretion of TNF-α even in SGC cells, as was seen with MGC cells, while DHA was the least effective fatty acid.

Similar to TNF-α, both MGC and SGC cells showed significant reduction in the secretion of VEGF in response to 5-FU + PUFAs. Once again, of all the fatty acids tested, GLA was found to be

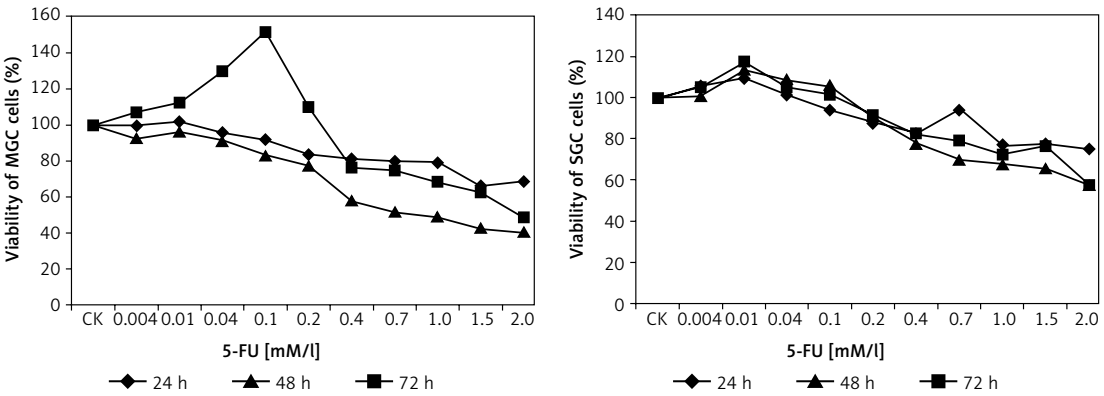

Figure 1. Effect of 5-FU alone on survival of gastric cancer cells *in vitro*. Three curves in each figure show proliferation trends for cells treated for 24 h, 48 h, and 72 h

Table I. Viability of MGC cells treated with 5-FU and PUFAs

PUFA	5-FU [mM]					
	0.004	0.04	0.1	0.2	0.4	0.7
None	0.99 ±0.02	0.97 ±0.02	0.93 ±0.02	0.79 ±0.02	0.68 ±0.03	0.58 ±0.02
ALA(L)	0.85 ±0.04	0.84 ±0.03	0.80 ±0.04	0.72 ±0.08	0.58 ±0.06	0.56 ±0.03
ALA(H)	0.78 ±0.02	0.79 ±0.03	0.75 ±0.03	0.64 ±0.09	0.62 ±0.02	0.58 ±0.04
DHA(L)	0.84 ±0.06	0.81 ±0.03	0.75 ±0.01	0.66 ±0.01	0.58 ±0.01	0.53 ±0.01
DHA(H)	0.80 ±0.01	0.80 ±0.02	0.72 ±0.03	0.64 ±0.03	0.60 ±0.01	0.54 ±0.02
EPA(L)	0.85 ±0.02	0.83 ±0.03	0.78 ±0.01	0.66 ±0.07	0.57 ±0.03	0.53 ±0.04
EPA(H)	0.78 ±0.06	0.78 ±0.03	0.71 ±0.02	0.65 ±0.03	0.55 ±0.03	0.50 ±0.02
LA(L)	1.01 ±0.03	1.00 ±0.06	0.87 ±0.02	0.76 ±0.02	0.65 ±0.01	0.59 ±0.03
LA(H)	0.95 ±0.04	0.94 ±0.02	0.93 ±0.05	0.80 ±0.01	0.70 ±0.02	0.62 ±0.05
AA(L)	0.88 ±0.02	0.88 ±0.02	0.80 ±0.02	0.68 ±0.02	0.55 ±0.01	0.52 ±0.01
AA(H)	0.85 ±0.01	0.87 ±0.01	0.80 ±0.02	0.68 ±0.02	0.56 ±0.02	0.49 ±0.04
GLA(L)	0.91 ±0.04	0.93 ±0.03	0.90 ±0.03	0.79 ±0.05	0.67 ±0.02	0.58 ±0.04
GLA(H)	0.88 ±0.03	0.92 ±0.02	0.84 ±0.06	0.77 ±0.02	0.65 ±0.02	0.61 ±0.02

Table II. Viability of SGC cells treated with 5-FU and PUFAs

PUFA	5-FU [mM]					
	0.004	0.04	0.1	0.2	0.4	0.7
None	1.01 ±0.05	0.95 ±0.09	0.90 ±0.13	0.69 ±0.10	0.65 ±0.05	0.59 ±0.09
ALA(L)	1.02 ±0.04	0.91 ±0.04	0.80 ±0.03	0.69 ±0.05	0.60 ±0.03	0.54 ±0.06
ALA(H)	0.96 ±0.04	0.92 ±0.05	0.75 ±0.09	0.63 ±0.04	0.54 ±0.04	0.52 ±0.03
DHA(L)	1.11 ±0.11	0.97 ±0.24	0.85 ±0.05	0.72 ±0.11	0.64 ±0.08	0.57 ±0.09
DHA(H)	0.98 ±0.03	0.95 ±0.02	0.77 ±0.02	0.64 ±0.04	0.52 ±0.01	0.54 ±0.06
EPA(L)	1.03 ±0.01	0.91 ±0.06	0.81 ±0.04	0.66 ±0.04	0.60 ±0.02	0.57 ±0.04
EPA(H)	1.07 ±0.08	1.01 ±0.09	0.82 ±0.06	0.61 ±0.03	0.52 ±0.06	0.51 ±0.06
LA(L)	1.04 ±0.05	0.96 ±0.08	0.81 ±0.04	0.71 ±0.03	0.63 ±0.05	0.56 ±0.01
LA(H)	1.10 ±0.04	0.97 ±0.03	0.88 ±0.08	0.76 ±0.06	0.66 ±0.11	0.66 ±0.12
AA(L)	1.11 ±0.06	1.02 ±0.03	0.77 ±0.05	0.63 ±0.06	0.54 ±0.06	0.50 ±0.04
AA(H)	1.02 ±0.08	0.93 ±0.07	0.78 ±0.05	0.61 ±0.11	0.49 ±0.09	0.48 ±0.07
GLA(L)	1.11 ±0.08	1.05 ±0.07	0.86 ±0.01	0.81 ±0.07	0.53 ±0.03	0.52 ±0.02
GLA(H)	1.11 ±0.06	0.93 ±0.04	0.78 ±0.06	0.70 ±0.06	0.63 ±0.05	0.61 ±0.05

Cell viability = $(OD_{treatment} - OD_{culture\ medium})/(OD_{control} - OD_{culture\ medium})$.

the most potent in suppressing VEGF secretion by both MGC and SGC cells *in vitro*.

Effect of 5-FU + PUFAs on LPL, PPAR-γ and C/EBP-α activity

Next, we tested the effect of 5-FU + PUFAs on the activities of LPL, PPAR-γ and C/EBP-α in MGC and SGC cells *in vitro*. These results, shown in Figures 4–6, revealed that there was a significant change in the levels of LPL, PPAR-γ and C/EBP-α in both MGC and SGC cells. For instance, it was found that LA and AA in combination with 5-FU produced the most significant increase in LPL activity in both MGC and SGC cells compared to all other fatty acids (Figure 4). On the other hand, AA

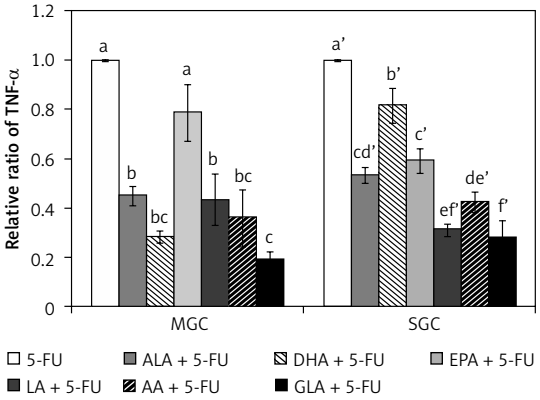

Figure 2. Relative ratio of TNF-α in gastric cancer cells treated with 5-FU and various PUFAs. (Relative ratio = concentration of TNF-α in gastric cancer cells treated with 5-FU + PUFAs/concentration of TNF-α in gastric cancer cells treated with 5-FU). For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 μM, 50 μM, 50 μM, 120 μM, 50 μM and 120 μM respectively; concentrations of 5-FU used were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, ALA, DHA, EPA, LA, AA, and GLA were 120 μM, 50 μM, 50 μM, 50 μM, 120 μM and 120 μM respectively; concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively

For MGC, a, b, c values in the same column with different letters are significantly different (p < 0.05); for SGC, a', b', c' values in the same column with different letters are significantly different (p < 0.05).

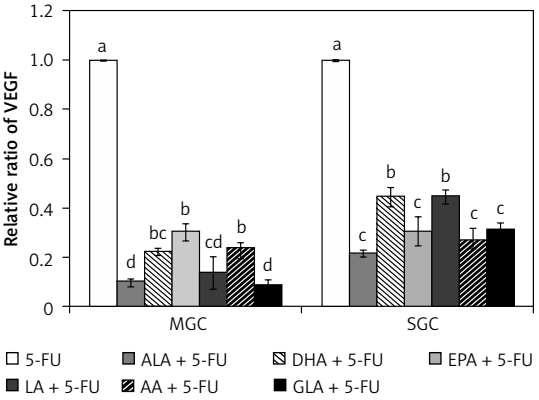

Figure 3. Relative ratio of VEGF in gastric cancer cells treated with 5-FU and various PUFAs. (Relative ratio = concentration of VEGF in gastric cancer cells treated with 5-FU + PUFAs/concentration of VEGF in gastric cancer cells treated with 5-FU). For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 μM, 50 μM, 50 μM, 120 μM, 50 μM and 120 μM respectively; concentrations of 5-FU were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, ALA, DHA, EPA, LA, AA, and GLA were 120 μM, 50 μM, 50 μM, 50 μM, 120 μM and 120 μM respectively; concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively

For MGC, a, b, c values in the same column with different letters are significantly different (p < 0.05); for SGC, a', b', c' values in the same column with different letters are significantly different (p < 0.05).

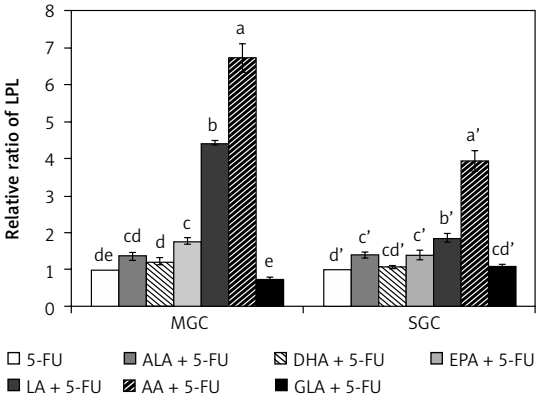

Figure 4. Relative ratio of LPL in gastric cancer cells treated with 5-FU and various PUFAs. (Relative ratio = concentration of LPL in gastric cancer cells treated with 5-FU + PUFAs/concentration of LPL in gastric cancer cells treated with 5-FU). For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 μM, 50 μM, 50 μM, 120 μM, 50 μM and 120 μM respectively; concentrations of 5-FU were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, ALA, DHA, EPA, LA, AA, and GLA were 120 μM, 50 μM, 50 μM, 50 μM, 120 μM and 120 μM respectively; concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively

For MGC, a, b, c, d, e values in the same column with different letters are significantly different (p < 0.05); for SGC, a', b', c', d' values in the same column with different letters are significantly different (p < 0.05).

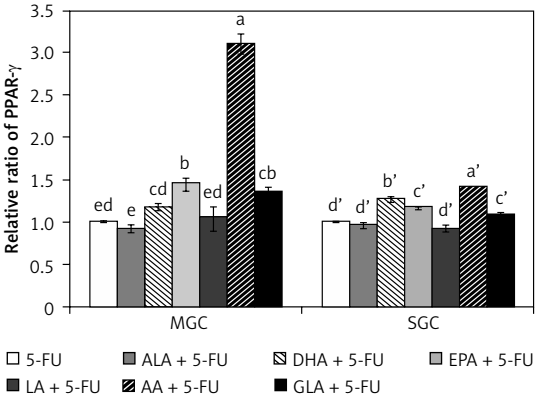

Figure 5. Relative ratio of PPAR-γ in gastric cancer cells treated with 5-FU and various PUFAs. (Relative ratio = concentration of PPAR-γ in gastric cancer cells treated with 5-FU + PUFAs/concentration of PPAR-γ in gastric cancer cells treated with 5-FU). For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 μM, 50 μM, 50 μM, 120 μM, 50 μM and 120 μM respectively; concentrations of 5-FU were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, ALA, DHA, EPA, LA, AA, and GLA were 120 μM, 50 μM, 50 μM, 50 μM, 120 μM and 120 μM respectively; concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively

For MGC, a, b, c, d, e values in the same column with different letters are significantly different (p < 0.05); for SGC, a', b', c', d' values in the same column with different letters are significantly different (p < 0.05).

is the only fatty acid that produced a significant increase in PPAR-γ activity in MGC cells but not in SGC cells (Figure 5). In contrast, both LA and AA in association with 5-FU induced a significant increase in the activity of C/EBP-α in both MGC and SGC cells.

Discussion

Previous studies showed that PUFAs possess selective tumoricidal actions both *in vitro* and *in vivo* [4–10]. It was noted that PUFAs enhance free radical generation and the lipid peroxidation process and thus bring about their tumoricidal action [5, 19–22]. It is noteworthy that radiation and many chemotherapeutic drugs enhance free radical generation and lipid peroxidation and thus bring about their anti-cancer actions [reviewed in 21]. This suggests that selective enhancement of free radical generation and the lipid peroxidation process in tumor cells could be employed as a strategy to eliminate them. In this context, it is noteworthy that both TNF-α and VEGF are not only pro-inflammatory molecules but are also capable of enhancing free radical generation [23–26]. The TNF-α enhances the production of VEGF and VEGF, in turn increasing that of TNF-α [27–32]. This implies that whenever there is an increase in the production of TNF-α or VEGF, automatically the synthesis and release of the other occurs. This is supported by the observation that, in general, cancer is associated with an increase in the plasma levels of both TNF-α and VEGF. In other words, if efforts are made to suppress the production of TNF-α or VEGF, it is expected that suppression of the synthesis of the other would also occur. The increased levels of TNF-α and VEGF are also expected to be responsible for the inflammatory reaction seen at the site of the cancer mass. In view of this close interaction between VEGF and TNF-α, we measured the production of both TNF-α and VEGF by the gastric tumor cells in response to PUFAs supplemented.

As expected, the results of the present study showed that various PUFAs suppressed the secretion of both TNF-α and VEGF by both MGC and SGC cells *in vitro* (Figures 2 and 3). Though all the fatty acids tested were effective in suppressing the secretion of both TNF-α and VEGF, GLA appears to be the most effective. To a large extent, AA was also as effective as GLA in suppressing the release of TNF-α and VEGF. These results are in agreement with the growth suppressive action shown by 5-FU + AA and 5-FU + GLA, though other fatty acids were almost as effective. For instance, the growth suppressive actions of various PUFAs in combination with 5-FU were as follows: when low-dose PUFAs were used, AA > DHA = EPA > ALA > GLA > LA, and when high-dose PUFAs were used,

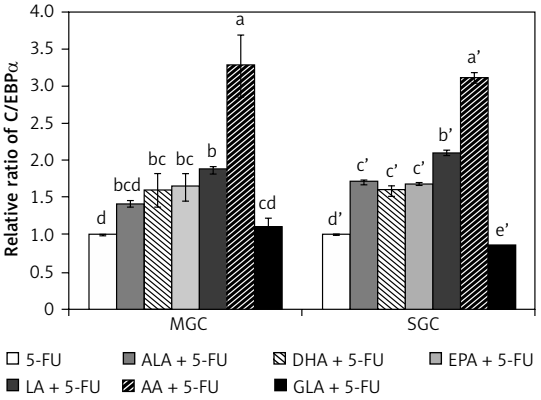

Figure 6. Relative ratio of C/EBPα in gastric cancer cells treated with 5-FU and various PUFAs. (Relative ratio = concentration of C/EBPα in gastric cancer cells treated with 5-FU + PUFAs/concentration of C/EBPα in gastric cancer cells treated with 5-FU). For MGC, concentrations of ALA, DHA, EPA, LA, AA and GLA were 120 µM, 50 µM, 50 µM, 120 µM, 50 µM and 120 µM respectively; concentrations of 5-FU were 0.2 mM, 0.2 mM, 0.2 mM, 0.7 mM, 0.4 mM, and 0.1 mM respectively. For SGC, ALA, DHA, EPA, LA, AA, and GLA were 120 µM, 50 µM, 50 µM, 50 µM, 120 µM and 120 µM respectively; concentrations of 5-FU were 0.1 mM, 0.1 mM, 0.1 mM, 0.1 mM, 0.4 mM, and 0.1 mM respectively

For MGC, a, b, c, d values in the same column with different letters are significantly different ($p < 0.05$); for SGC, a', b', c', d', e' values in the same column with different letters are significantly different ($p < 0.05$).

AA > EPA > DHA > ALA > GLA > ALA for MGC cells; whereas when low-dose PUFAs were used, AA > GLA > ALA > LA > EPA = DHA, and when high-dose PUFAs were used, AA > GLA > EPA > ALA > DHA > LA for SGC cells. Thus, AA is the best growth suppressive fatty acid with regard to both SGC and MGC cells, irrespective of the dose of fatty acid employed. The second best growth suppressive fatty acid is EPA or DHA for MGC cells, whereas GLA is the second best fatty acid when SGC cells are taken into consideration.

On the other hand, GLA was the most effective fatty acid in suppressing the secretion of both TNF-α and VEGF by MGC and SGC cells. The next best fatty acid to suppress the secretion of TNF-α and VEGF by both MGC and SGC cells is AA. Thus, it is the n-6 fatty acid AA that is the most effective fatty acid both in terms of growth suppression and inhibition of VEGF and TNF-α secretion.

The PUFAs being lipids, it is expected that they could have an effect on lipid metabolism related factors such as LPL, PPAR-γ and C/EBP-α. Studies done in this regard clearly showed that all the PUFAs tested could have considerable action on LPL, PPAR-α and C/EBP-α. Of all the fatty acids tested, AA induced the highest increase in the levels of LPL, PPAR-α and C/EBP-α in both MGC and SGC cells. Since AA is the most effective growth

suppressive fatty acid and the one that produced a significant decrease in VEGF and TNF-α secretion in these cells, it is tempting to propose that a combined action on all these indices is responsible for its tumoricidal action observed in the present study.

Lipoprotein lipase gene and enzyme activity were found to be increased in lung cancer tissue, musculoskeletal sarcomas, and abdominal subcutaneous tissue of subjects with advanced gastric and esophageal cancer [33–35]. High LPL activity in cancer tissue provides a possible mechanism for increasing the supply of lipid nutrients to the tumor that is necessary for tumor growth. For instance, it was reported that compared with controls or non-responding tumors, expression of PPAR-γ and LPL activity was elevated in those that responded to LGD1069, a high-affinity ligand for the retinoid X receptors in a rat mammary tumor model, suggesting that tumor regression involves differentiation induction along the adipocyte lineage [36]. Furthermore, it was reported that LPL, in the presence of triglyceride-rich lipoproteins, accelerates the growth of breast cancer and sarcoma cells [37]. It was also noted that providing LPL to prostate cancer cells, which express low levels of the enzyme, did not augment growth, but did prevent the cytotoxic effect of fatty acid synthesis inhibitors. Moreover, LPL knockdown inhibited HeLa cell growth. These findings indicate that, in addition to *de novo* lipogenesis, cancer cells use LPL to acquire fatty acids from the circulation by lipolysis, and this can fuel their growth.

In this context, it is interesting to note that TNF-α has a bifunctional regulatory nature of both inhibiting tumor cell proliferation and enhancing growth and inducing their differentiation under some very specific conditions. The TNF-α is believed to be an essential local and systemic mediator of cancer cachexia. In those with infections, the action of TNF-α on LPL activity to enhance the secretion of free fatty acids from adipocytes is probably important to provide additional energy needed by the immune system to combat invasion. However, the relationship between the ability of TNF-α to suppress the anabolic processes and its cytotoxic action on tumor cells and their relationship to produce cancer cachexia remains undetermined. In the adipocytes, TNF-α suppresses LPL, which leads to a reduction in TG uptake and lipid deposition, contributing to cachexia [38, 39]. Hence, it has been suggested that neutralizing the action of TNF-α may reverse the cachectic processes and potentially improve the general condition of patients with cancer. But, subsequent studies using both wild-type and gene-deficient mice for TNF-α receptor type 1 protein revealed that implantation of Lewis lung carcinoma resulted in a considerable loss of carcass weight in both these groups, with the wild-type mice showing loss of both fat and muscle, whereas the TNF-α receptor type 1 gene-knockout mice showed much less muscle wastage though both groups of animals showed a significant increase in circulating TNF-α. Muscle wastage in wild-type mice was found to be due to an increase in protein degradation with no changes in protein synthesis, which results in a decreased rate of protein accumulation, accounting for the muscle weight loss observed as a result of the tumor burden. In contrast, gene knockout mice did not show significantly lower rates of protein accumulation as a result of tumor implantation [40]. These results suggest that TNF-α is responsible for protein breakdown in skeletal muscle of tumor-bearing mice and has much less effect on adipose tissue loss. Subsequent studies revealed that TNF-α has a biphasic activation of NF-κB (NF-κB) in differentiated skeletal muscle cells wherein the first transient phase was terminated within 1 h of cytokine addition, while the second phase persists for an additional 24–36 h [41]. Biphasic activation is mediated at both the levels of NF-κB DNA binding and transactivation function, and both phases are dependent on the IKK/26 S proteasome pathway. It was reported that regulation of the first transient phase is mediated by the degradation and subsequent re-synthesis of IκBα, whereas the second phase activity correlated with persistent down-regulation of both IκBα and IκBβ proteins due to a continuous TNF signal. Importantly, inhibition of NF-κB prior to initiation of the second phase of activity inhibited cytokine-mediated loss of muscle proteins [41]. These results implicate the biphasic activation of NF-κB in response to TNF in skeletal muscle wasting associated with cancer/infection associated cachexia.

Decreased albumin expression is common in patients who develop cachexia due to chronic diseases, including cancer. In a simulation of this model, TNF-α treatment of primary mouse hepatocytes or TNF-α overexpression in a mouse model of cachexia induced oxidative stress, nitric oxide synthase (NOS) expression and phosphorylation of C/EBPβ on Ser239, within the nuclear localization signal, thus inducing its nuclear export, which inhibits transcription of the albumin gene. 3-Morpholinosydnonimine (SIN-1), an NO donor, duplicated the TNF-α action on hepatocytes. It is interesting that similar molecular abnormalities were found in the liver of patients with cancer cachexia. Treatment of TNF-α-treated mice with antioxidants or NOS inhibitors prevented phosphorylation of C/EBPβ on Ser239 and its nuclear export, and rescued the abnormal albumin gene expression. This suggests that TNF-α suppressed

Table III. Summary of results obtained on the effect of 5-FU + PUFAs on the activities/levels of TNF-α, VEGF, LPL, PPAR-γ and C/EBPα

Variable		Dose of 5-FU used [mM]	Relative ratio				
			TNF-α	VEGF	LPL	PPAR-γ	C/EBPα
MGC	120 µM ALA + 0.2 mM 5-FU	0.2	0.45 ±0.04	0.10 ±0.01	1.37 ±0.11	0.92 ±0.04	1.42 ±0.04
	50 µM DHA + 0.2 mM 5-FU	0.2	0.28 ±0.03	0.22 ±0.02	1.23 ±0.09	1.18 ±0.05	1.60 ±0.22
	50 µM EPA + 0.2 mM 5-FU	0.2	0.79 ±0.12	0.30 ±0.03	1.79 ±0.10	1.46 ±0.08	1.65 ±0.19
	120 µM LA + 0.7 mM 5-FU	0.7	0.43 ±0.11	0.14 ±0.07	4.42 ±0.07	1.05 ±0.15	1.89 ±0.05
	50 µM AA + 0.4 mM 5-FU	0.4	0.36 ±0.11	0.23 ±0.03	6.73 ±0.40	3.11 ±0.12	3.28 ±0.41
	120 µM GLA + 0.1 mM 5-FU	0.1	0.19 ±0.04	0.08 ±0.02	0.75 ±0.04	1.37 ±0.06	1.11 ±0.12
SGC	120 µM ALA + 0.1 mM 5-FU	0.1	0.53 ±0.03	0.22 ±0.01	1.39 ±0.08	0.97 ±0.03	1.72 ±0.04
	50 µM DHA + 0.1 mM 5-FU	0.1	0.82 ±0.07	0.45 ±0.04	1.09 ±0.05	1.27 ±0.04	1.60 ±0.07
	50 µM EPA + 0.1 mM 5-FU	0.1	0.59 ±0.05	0.31 ±0.06	1.39 ±0.14	1.17 ±0.02	1.69 ±0.02
	50 µM LA + 0.1 mM 5-FU	0.1	0.31 ±0.02	0.45 ±0.03	1.86 ±0.12	0.93 ±0.04	2.10 ±0.04
	120 µM AA + 0.4 mM 5-FU	0.4	0.42 ±0.04	0.27 ±0.05	3.94 ±0.27	1.41 ±0.02	3.12 ±0.08
	120 µM GLA + 0.1 mM 5-FU	0.1	0.28 ±0.07	0.31 ±0.03	1.09 ±0.06	1.09 ±0.03	0.85 ±0.01

albumin gene expression (and thus inhibits albumin synthesis) by phosphorylation of C/EBPβ and enhanced the synthesis of NO [42]. This coupled with the observation that C/EBPβ can functionally replace C/EBPα in the liver but not in adipose tissue is not only interesting [43] but also suggests that the ability of TNF-α to suppress albumin production is due to its action on C/EBPβ in the liver and induction of lipolysis is by acting on C/EBPα in adipose tissue.

In this context, it is interesting to note that PPAR-γ inhibited NO and TNF-α synthesis [44] and has anti-inflammatory and anti-proliferative actions [45]. These results emphasize the close interaction(s) among TNF-α, LPL, PPAR-γ and C/EBPα and C/EBPβ.

In the present study, we observed that both LA and especially AA in association with 5-FU enhanced LPL, PPAR-γ and C/EBPα activities in both MGC and SGC cells compared to the control (see Figure 4 and Table III for comparison of actions of various PUFAs on TNF-α, LPL, PPAR-γ and C/EBPα activities). These actions may explain, to some extent, the anti-inflammatory, anti-cachectic and anti-tumor activities of PUFAs. Other PUFAs did enhance the LPL activity and that of PPAR-γ and C/EBPα but were not as potent as AA (Figures 4–6). The action of PUFAs on PPAR-γ and C/EBPα may explain their inhibitory action on the production of TNF-α and VEGF noted in the present study.

In conclusion, PUFAs have anti-cancer actions and are capable of potentiating the tumoricidal action of the anti-cancer drug 5-FU on gastric cancer cells *in vitro*. Based on the results of the present study, it is reasonable to propose that anti-cancer, anti-inflammatory and anti-cachectic properties of PUFAs are due to their action on TNF-α, VEGF, LPL, PPAR-γ, and C/EBPα and thus may be useful to manage cancer-associated hyperlipidemia [46] and could be used in combination with other agents to induce apoptosis of various cancer cells, especially of gastric tumor cells [47].

Conflict of interest

The authors declare no conflict of interest.

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