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Delay of Lung Adenocarcinoma (LAC-1) Development in Mice by Dietary Oleic Acid

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ABSTRACT

Lung cancer is the leading cause of death by cancer, and is a major sanitary concern worldwide. Some nutrients, such as ω-9 fatty acids, have been proposed as anticancer agents. Thus, an olein-enriched diet was assayed in a murine model of lung adenocarcinoma (LAC-1) to evaluate neoplastic and paraneoplastic evolution in BALB/c mice. The organic assimilation of dietary fatty acids was confirmed in liver by gas chromatography. This experimental oleic acid-containing diet increased animal survival and tumour latency (analysed by the Kaplan-Meier method), improving neoplastic evolution and general status, with weak effects on the paraneoplastic syndrome (thymus atrophy, splenomegaly, splenocyte response to mitogen, blood anaemia, and leucocytosis). Tumour lipid oxidation was not involved. Thus, diet enrichment with olein, a natural source of the ω-9 oleic acid, significantly delayed progression of LAC-1 and increased tumour latency and mice survival. These results support its use in nutritional management of cancer, with further studies being encouraged.

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Introduction

Lung cancer is the worldwide leading cause of oncological death with poor prognosis, in both developed and developing countries (1). Concerning this, epidemiological and experimental data have shown that nutritional factors may be considered as major epigenetic determinants, which modulate tumour development (2). These nutrients can modify cancer cell biology and the organic response, to control pathological evolution (3).

An important group of potential bioactive nutrients is constituted by dietary monounsaturated ω-9 fatty acids, such as the oleic acid, which have been scarcely studied in lung carcinogenesis (4). Moreover, contradictory results exist because diet with high content of oleic acid reduces bioavailability of essential fatty acids, which in turn can cause the pro-tumour condition called essential fatty acid deficiency (5). Nonetheless, lung cancer depends on an intricate network of pathological factors, which include the immune response (6). Thus, unidirectional conclusions cannot be made from experimental outcomes.

Consequently, our aim was to evaluate the modulating effects of dietary oleic acid on cancer progression, using the previously developed murine model LAC-1 of transplantable lung adenocarcinoma (7).

Materials and Methods

Chemicals

Standard diet was from GEPSA (Argentina). Olein with 80% of ω-9 18:1 (oleic acid) was obtained from Retienne (Argentina). Solvents were purchased from Cicarelli (Argentina), and other chemicals were obtained from Sigma-Aldrich Inc. (USA).

Experimental Conditions

Murine transplantable lung adenocarcinoma LAC-1 was used in BALB/c mice, in accordance with a previous work (7). LAC1 is a tumour with a fast evolution and good metastatic capacity that induces a paraneoplastic syndrome characterized by anaemia, neutrophilia, cachexia, splenomegaly, and thymus atrophy (7).

Weanling BALB/c mice of both sexes (Age: 1 mo) were randomly distributed in two diet groups to receive: standard diet (Control Group, CG) or 6% olein-enriched diet (oleic acid group, OG), for 2.5 mo before tumour transplantation (Age: 3.5 mo), with food and water *ad libitum*. These feeding conditions were maintained until the end of the experiments.

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Commercial standard diet is considered as a normal diet for rodents with 6% fat (CG). The oleic acid group (OG) was fed on a semisynthetic diet matched with nutritional content of CG (both diets did not differ in their caloric values per gram). Its composition included: 6% olein, 17% casein, 33% sucrose, 38% corn starch, 2% fibre, 2% salt mixture, and 0.5% vitamin mixture (2). Fatty acid composition of the diet formulas is shown in Table 1. OG diet was weekly prepared and stored in dark sealed glass containers at 4°C until use (supply: three times a week with discard of the remnant).

A total number of 105 mice were used. They were separated into two diet groups, CG with 53 animals and OG with 52 ones.

The animals in every diet group were so separated in two groups, with or without tumour (38 and 15 animals respectively in CG and 34 and 18 in OG).

Animals were inspected daily and weighted weekly, under light anaesthesia as is usually done in our laboratory, with all procedures responding to ethical concerns and good laboratory practices (8).

Fatty Acid Analysis

Extraction of total lipids of liver and diets and analysis of methylated fatty acids by gas-liquid chromatography were performed as previously reported (9).

Tumour-related Parameters and Pathological Evolution

Time of Tumour Latency

It is defined as the period from transplant to palpable detection of the tumour.

Tumour Diameter

It was established once a week with a digital device by three cross-perpendicular measures.

Post-transplantation Survival Time

It was the period from tumour transplant until pathological endpoints (spontaneous death, or wasting syndrome with marked physical distress).

Table 1. Fatty acid composition of the utilized dietary formulas.*

	Saturated			Monosaturated		Polyunsaturated		
	16:0	18:0	20:0	16:1 ω7	18:1 ω9	18:3 ω3	18:2 ω6	20:4 ω6
CG	26.5	8.5	1.8	1.6	44.1	3.7	10.6	3.2
OG	4.0	4.0	–	7.7	**80.8**	0.4	3.0	—

*Percentage composition. Oleic acid is in bold.

Other Findings During Necropsy

Final tumour diameter, tumour weight, and final animal weight (after tumour extraction) were recorded (8).

Organ and Tissue Evaluation

Tumours and lymphoid organs (spleen and thymus) were weighted and analysed macroscopically and microscopically. Microscopic structures were evaluated using haematoxylin and eosin staining and light microscopy (histopathology). Lung metastases were measured and located to classify five grades: 0 (none), 1 (microscopic metastases), 2 (isolated macroscopic foci, 1–7 mm), 3 (confluent macroscopic foci occupying, at least, a major lung lobe), and 4 (confluent macroscopic focus occupying almost all lung parenchyma). Turk-stained blood cells were counted in the Neubauer chamber.

Assessment of ex vivo Response to Concanavalin A of Splenocytes

Spleen Cell Processing

After death, spleens were promptly removed aseptically, homogenized and placed in sterile RPMI-1640 medium with L-glutamine and without phenol red. The resultant cellular suspension was processed with a lysing buffer of red blood cells. Aliquots, containing 200,000 viable cells in RPMI −1640 medium with 13% of autologous serum, were seed in triplicate in 96-well plates. Cells were stimulated by the addition of concanavalin A (ConA) (2 or 5 μg/mL); control cultures received the same volume of medium without the mitogen. Plates were maintained for 72 h at 37°C in an atmosphere with 5% carbon dioxide.

Cell Culture Evaluation

The quantification of viable cells was performed using the MTT assay modified according Graber and Losa (10), after 72 h of culture. The formazan crystals formed were dissolved in anhydrous isopropyl alcohol-HCl (0.04 N). The number of viable cells was calculated from the difference in absorbance at 570–630 nm. The increase in the number of viable cells in the presence of ConA was considered as indicative of proliferation (11,12), whereas the decrease was indicative of cell death. The viability without ConA was basal viability in both animals with and without tumour.

Tumour Lipid Oxidation

Representative samples of tumours were separately homogenized. Each suspension (150 μL) was mixed with 750 μL of chloroform/methanol (2:1 v/v) for the extraction of total lipids (13). The mixture was centrifuged at 1,000 g for 10 min, and then the upper layer was eliminated. The lower layer was washed twice with chloroform/methanol/water

Table 2. Hepatic fatty acid profile in treated mice.*

	Saturated				Monosaturated			Polyunsaturated					
	14:0	15:0	16:0	18:0	14:1	16:1 ω7	18:1 ω9	18:3 ω3	20:5 ω3	18:2 ω6	20:4 ω6	22:4 ω6	20:3 ω9
CG	0.43	0.1	30.4	17.9	0.2	4.3	24.5	0.5	0.3	14.1	5.7	0.5	0.2
OG	0.34	—	16.7	5.3	—	9.4	54.8	1.2	0.1	6.2	3.3	0.3	2.4

*Percentage for animals fed with standard diet: CG [Triene (ω9)/Tetraene (ω6) ratio = 0.04], or olein: OG [Triene (ω9)/Tetraene (ω6) ratio = 0.71] diets.

(3:48:47 v/v/v) in sufficient amounts to obtain initial 900 μL. Finally, the resulting lower layer was dried under a nitrogen flow at room temperature. The lipids were resolubilized in 200 μL of ethanol and read against ethanol to record absorbance at 234 nm. Conjugated dienes (CD) were determined under low-temperature conditions according to Recknagel and Glende (14). Results were expressed as relative units compared to control samples.

Statistical Analysis

Data were reported as means ± standard deviation of $n > 3$. Differences between treatments were established by the t-test with a P value < 0.05. Spearman coefficients were calculated to assess variable correlations. Also, the cumulative probability of pathological outcomes in time was estimated by the Kaplan-Meier method, with diet differences being investigated with the log-rank test (15,16). Analyses were performed with the Infostat 2012 software (17).

Results

Fatty Acid Supply

Lipid profile and oleic acid food content were confirmed by gas chromatography in diets of CG and OG. Increased levels of oleic acid with less saturated and polyunsaturated acids were seen in OG (Table 1).

The organic incorporation of dietary fatty acids by mice was assessed in murine liver, with OG showing higher hepatic levels of oleic acid (Table 2).

Pathological Evolution

Tumour-bearing mice lost body weight. The OG lost 11.4% whereas the CG lost 30.7% of body weight respectively, with significant differences between them ($P = 0.0046$). Degree of tumour firmness was greater in case of CG, whereas it was fluctuating in OG. This was consequent with the solid tumours versus the necrotic ones found respectively by macroscopic assessment during the autopsies (data not shown).

Although animal death happened when tumours reached similar diameters, in both experimental groups (CG: 28.1 ± 1.2 mm, OG: 29.2 ± 1.1 mm) (Table 3), OG showed longer survival time and delayed tumour growth than CG ($P < 0.05$). In this regard, the diameter was a marker of bad prognosis, given the inverse > 50% correlations with survival time, thymus weight, and mitogenic response of splenocytes. Furthermore, OG-induced delay of cancer development was mainly determined by an increase of latency with respect to CG ($P < 0.05$; Fig. 1 and Table 3). Although tumour growth progression was lower in OG than in CG at the fifth and sixth weeks after LAC-1 transplant ($P = 0.0138$ and $P = 0.0416$, respectively) (Fig. 2), time progression was similar in both groups after first tumour palpation (CG: 40.26 ± 4.39 days, OG: 42.57 ± 7.45 days) (Table 3).

Table 3. Tumour-related parameters.

	Time of tumour latency	Final tumour diameter	Post-transplantation survival time
CG	10.69 ± 0.69 days*	28.1 ± 1.2 mm	50.61 ± 4.65 days#
OG	13.75 ± 1.36 days*	29.2 ± 1.1 mm	60.28 ± 8.77 days#

*Significant difference $P = 0.008$. #Significant difference $P = 0.036$.

On the other hand, incidence of pulmonary metastasis was similar in both dietary groups by affecting 58% of

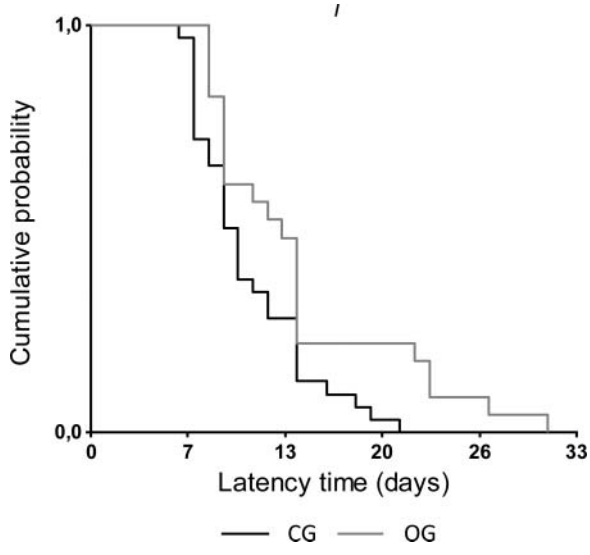

Figure 1. Latency time of tumour-bearing mice fed with standard diet (CG) or with olein-enriched diet (OG). Time curves were generated by the Kaplan-Meier method and diet differences were investigated with the log-rank test (Chi square = 4.133, $P = 0.042052$).

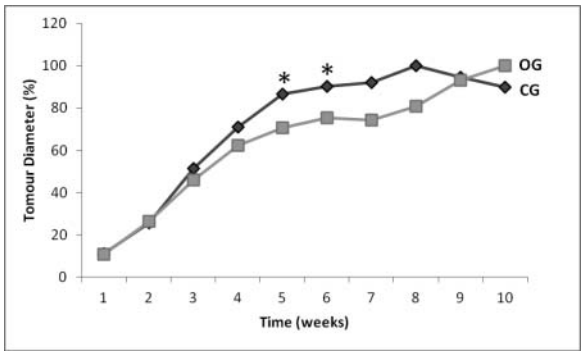

Figure 2. Tumour growth progression in mice fed with standard diet (CG) or with olein-enriched diet (OG). Graphic represents a linear tendency for each dietary group. *Significant differences at fifth and sixth week after tumour transplant ($P = 0.0138$ and $P = 0.0416$, respectively). Animal number per group: at tumour transplantation $n \geq 30$; at experiment end $n \geq 5$. Tumour growth was expressed to the widest diameter reached during tumour evolution.

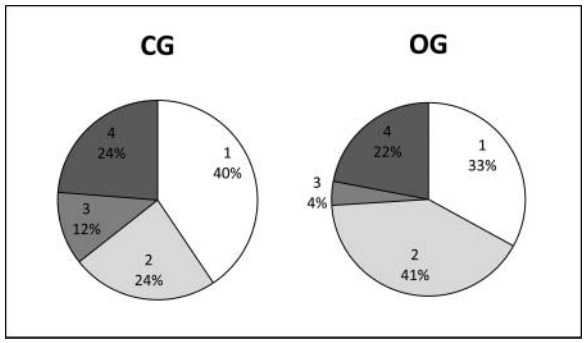

Figure 3. Degrees of metastasis in tumour-bearing animals with metastasis.

cases, as follows: 41% presented small–intermediate spread (grades 1 and 2), 17% showed advance metastasis (grades 3 and 4) (Fig. 3).

Biological Response

Other concordant non-significant biological results found in the OG were higher liver weight (CG: 1.5 ± 0.1 g, OG: 1.8 ± 0.1 g) (Table 4), and blood erythrocyte count (CG: $5.46 \times 10^6 \pm 0.76 \times 10^6$ cells/mm^3, OG: $6.04 \times 10^6 \pm 0.49\ 10^6$ cells/mm^3) (Fig. 4), with lower blood leukocyte count (CG: $10.98 \times 10^3 \pm 3.27 \times 10^3$ cells/mm^3, OG: $7.41 \times 10^3 \pm 2.25 \times 10^3$ cells/mm^3) (Fig. 5).

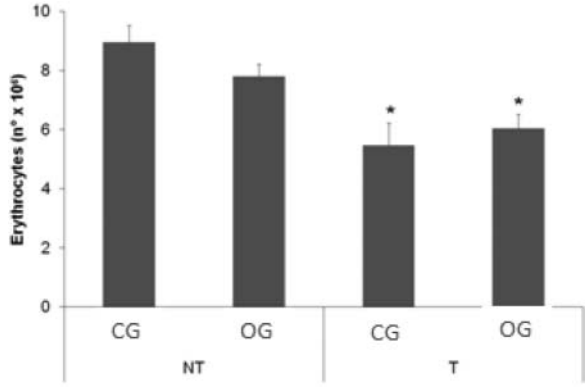

Figure 4. Erythrocyte count (per mm^3) from animals with tumour (T) or without it (NT) under standard or olein-enriched diets (CG and OG, respectively). *Mean ± SE ($n > 3$), significant decrease ($P < 0.05$).

The thymus weight, marker of better prognosis (inverse > 50% correlations with metastasis incidence, tumour diameter and blood leukocyte count, but positive correlations with final body weight and blood erythrocyte count), was reduced in 97% of tumour-bearing mice (atrophy: 20.0 ± 1.4 mg) (Table 4), in both diet groups. On the other hand, tumour-bearing mice showed splenomegaly (spleen weight: 0.4 ± 0.1 g) (Table 4).

No significant differences were found in mitogenic response of splenocytes from CG (100.0 ± 4.7% respect to non-tumour-bearing mice, with ConA concentration adjusted) and OG (143.1 ± 6.9%). This was concordant with the absence of microscopic differences in neoplastic parenchyma and invasion by lymphocytes (data not shown).

Levels of CD, markers of lipid oxidation were similar in both dietary treatments (CG: 100 ± 16%, OG: 101 ± 15%).

Discussion

Olein was a relevant nutritional source of oleic acid for mice. After intake, hepatic cells receive circulating fatty acids and incorporate them into their membranes (18,19). When a specific fatty acid is supplied in significant amounts, it is highly available for these cells, which possess several mechanisms to metabolize it (18). Thus, oleic acid was found in liver, in accordance with other authors (20).

Table 4. Fatty acid composition of the utilized dietary formulas.*

	Thymus (mg)		Spleen (g)		Liver (g)	
	NT	T	NT	T	NT	T
CG	33.86★ ± 4.43	9.44★ ± 1.85	0.16• ± 0.02	0.51• ± 0.14	1.43 ± 0.07	1.53 ± 0.1
OG	37.79# ± 2.21	11.22# ± 2.26	0.11* ± 0.01	0.57* ± 0.23	1.32 ± 0.04	1.75 ± 0.1

NT: control without tumour. T: with tumour. ★and #$P < 0.0001$ indicative of thymus atrophy. •and *$P < 0.0001$ indicative of splenomegaly.

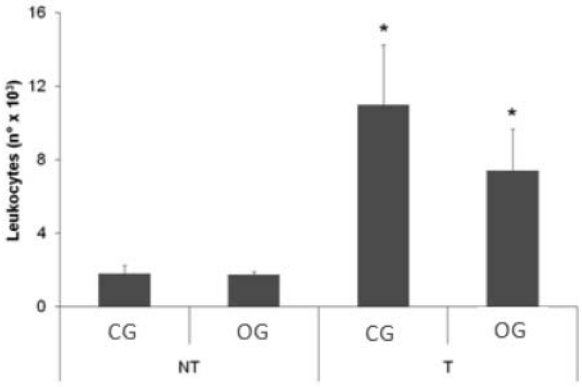

Figure 5. Leukocyte count (per mm^3) from animals with tumour (T) or without it (NT) under standard or olein-enriched diets (CG and OG, respectively). *Mean ± SE ($n > 3$), significant increase ($P < 0.05$).

Given that immune and oxidative markers did not differ significantly between experimental groups, it is suggested that diets directly modulated the primary tumour, with the immune paraneoplastic syndrome of LAC-1 being resistant to the assayed nutritional intervention (7). Moreover, this modulation could improve the general status of tumour-bearing mice, which was significant in case of OG. Oleic acid regulates cancer physiology by several mechanisms, such as apoptosis induction, mitosis arrest, and cellular differentiation (21). Also, it impairs formation of cancer vessels (21,22), which might explain necrosis and its prolonged latency and progression of tumours found in OG. Angiogenesis is indispensable for cancer implant and growth; thus, it is an important target in oncology (23).

Numerous works support the importance of targeting cancer development at early stages to obtain better therapeutic outcomes (23–25), as confirmed here concerning diet. After tumour appearance, control of its diameter should be also considered to identify appropriately neoplastic evolution and its nutritional modulation, as the diameter depends on tissue integrity (26), i.e. decreased neovascularization leads to necrosis and reduced tumour diameter, thus enhancing survival. Consequently, belated treatments are ineffective to control cancer. In fact, advanced metastasis and organic compromise were not prevented in the current work.

Concerning systemic compromise, lung metastases were expected given the tissue origin of LAC-1 and the homing phenomenon of their cells (7). On the other hand, this illness causes an immunopathological paraneoplastic syndrome, which includes thymus atrophy, splenomegaly, anaemia, leucocytosis, and impaired T cell response (7). Although oleic acid exhibits immunomodulatory capacity (27), the OG did not show further improvement of the immune paraneoplastic syndrome. However, this experimental group presented delayed tumour progression and increased survival.

Data about dietary monounsaturated fatty acids are not always conclusive (28–30). In this sense, although a detrimental pro-tumour effect of essential fatty acid deficiency has been described (31), diet enrichment with oleic acid improved animal resistance against the studied lung cancer. Consequently, protective effects of olive oil-containing diets have been reported (28), with oleic acid being the main monounsaturated fatty acid of olive oil (32). In agreement with other authors (29,33), our results indicated that oleic acid had anticancer properties and extended pathological latency.

Conclusion

Diet enrichment with olein, a natural source of ω-9 oleic acid, significantly delayed progression of the lung adenocarcinoma LAC-1, leading to increased tumour latency and mice survival. Thus, these results support its use in lung cancer control, with further mechanistic studies being encouraged.

Acknowledgments

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Conflicts of Interest

The authors declare that it represents an original work, which has not been previously published and is not currently being considered by another journal. If it is accepted for your prestigious Journal, it will not be published elsewhere in English or in any other language without the Editor's consent.

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