Communication

Preliminary Data: Feeding and Colon Cancer Growth. Modified Nitrogen Intake Leads to Blunted Cancer Growth Independently by Serine

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Abstract: Cancer cells are originated by normal cells and with those share the need to be fed. Metabolic reprogramming in cancer cells is not yet fully understood, and there is a vast literature suggesting that any macronutrient may be efficiently utilized by cancer for duplication purposes. Metabolic hyperactivity of cancer and continuous duplication requires huge amounts of both energy and substrates for synthesis of components of new cells, and the modifications of metabolism induced by cancer cells to survive and grow. In rodents, we have recently observed that modifications of the normal diet, obtained by increasing the percentages of essential to non-essential amino acids (EAA/nonEAA), resulted in improved lifespans. *In vitro*, EAA supplementation promoted colon cancer cells apoptosis by enhanced autophagy. Therefore, we tested in mice if injection of colon cancer cells would have been followed by unvaried development of cancer volumes in animals fed with standard laboratory diet (controls) versus EAA rich modified diets (EAArmd). Both diets perfectly matched in total macronutrients content and concentration. Results of the first set of 8 animals, 4 controls and 4 EAArmd-fed, showed that controls develop cancers nearly 5 times larger and heavier than EAArmd-fed (P<0,002). We discuss which epigenetic modifications would be involved and worth to be studied, and what kind of scenarios those preliminary findings, if confirmed, would open.

Keywords: cancer; amino acids; colon; nitrogen, diet, mice

1. Introduction

We have recently reviewed literature focusing on calories, carbohydrates, lipids and proteins/amino acids ratios provided by food to cancer bearing patients [1]. We also suggested the existence of a possible energy dependent relationship (ruling reciprocal dimensions of synthesis) between high energy consuming processes, and autophagy, whose activation lowers ATP levels thus increasing AMP concentration. The attendant AMP-kinase (AMPK) activation [1] blunts mTORC1 (mammalian target of rapamycin) dependent protein synthesis and activates the autophagic machinery, promoting ATP refueling in continuous synchrony [2]. We previously showed *in vitro* that autophagy is triggered by providing to cancer cells an excess of EAA [3].

Other experimental reports have formerly described the effects on cancer cells of heightened EAA concentration [4, 5], although in Methods it was not openly recognized that by subtracting from tested formulations some non-EAA (serine and glycine), obviously EAA percentages would have been increased, as lately acknowledged [6] and

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discussed elsewhere [7]. In the present study, we investigated whether altering significantly EAA/non-EAA ratios in diets, would influence *in vivo* cancer development. Furthermore, we investigated the role of serine in cancer including such amino acid in the non-EAA smaller fraction [7].

2. Results

These preliminary data suggest that, among macronutrients, AA "quality" is the most determinant factor in cancer growth promoting a conspicuous slowdown in the growth of the subcutaneous tumor in animals fed with the EAArmd. Photos at the site of injection and explanted tumor mass in one sample of any group are presented in Figure 1

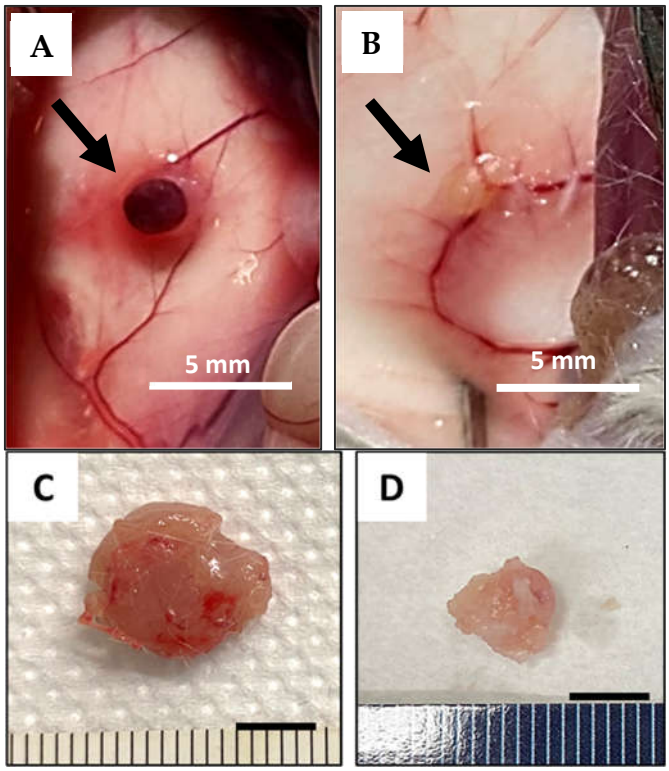

Figure 1. **A-B**: photograph of the inner surface of the flank region where the tumor cells were injected (arrow) in animals fed with StD (**A**) and fed with EAArmd (**B**). **C-D**: example of explanted subcutaneous tumor mass in animals fed with StD (**C**) and fed with EAArmd (**D**) after 21 days. Scale bar 5 mm.

Rough data of cancers volumes (mm^3) [(maximal diameter x smallest $diameter^2$)/2], and weights (grams, in brackets) are presented in **Table 1.**

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Table 1. Volumes of cancers (mm^3) after 3 weeks (21 days) from subcutaneous inoculation of CT26 cancer cells in mice fed with StD and EAArmd respectively. In parentheses, weights in grams. T-test, *p<0.01

	Cancer volume and [**weight**]	
	StD (EAA/non-EAA <0,9)	**EAArmd** (EAA/non-EAA <5)
Mouse 1	473.8 [0.39]	112.5 [0.12]
Mouse 2	447.8 [0.21]	68.8 [0.09]
Mouse 3	400 [0.20]	135 [0.13]
Mouse 4	462.5 [0.32]	67.5 [0.11]
Sum of volume (mm^3)	1784.1	383.8 *

Statistical analysis was performed by Student's T test for paired data, two tail calculation, type 1, and standard regression test by Microsoft Office Excel. In both groups, tumor volume and weight are correlated (r = 0,83 and 0,94 respectively). Sum of volumes (1784.1 in controls and 383.8 in EAArmd) showed a ratio (4.65) strikingly most elevated in control (low EAA/non-EAA) animals. By T-test, differences between volumes in the two groups were confirmed (p<0.0012) as well as those between weights (p<0.03).

3. Discussion

Our preliminary data suggest that, among macronutrients, AA "quality" is a most determinant factor in cancer growth. Indeed, as already observed *in vitro* [3], altering EAA/non-EAA ratios (which is regularly <0.9) may have a deep impact on cancer biology [7]. Such ratio can only be efficiently maintained *in vivo* by proteolysis, whose intervention releases an excess of non-EAA, matching the EAA supplied by EAArmd diet. Such compensatory mechanism cannot be established *in vitro* where EAA supplied to cells keep dominating the nutritional fluid composition without any chance to compensate such imbalance. Also, this study suggests that AA ratios provided by food are a most efficient determinant in eliciting modifications of metabolism in cells. Hence, macronutrients should not be considered just passive molecules used by cells according to their metabolic needs. Indeed, food quality appear to modify cell behaviors actively and consistently. Thus, epigenetic modifications, such as changes in EAA/non-EAA ratios, can be implemented and maintained as long as necessary by engineering food compositions. Conversely, subtraction of non-EAA (single or pairs) such as serine and glutamine would be an inefficient strategy unless resulting in a rise of EAA/non-EAA ratio [7].

4. Materials and Methods

The experimental protocol was approved and conducted in accordance with laws of the Italian Ministry of Health and complied with the 'The National Animal Protection Guidelines'. The Ethical Committee for animal experiments of the University of Brescia (OPBA) and the Italian Ministry of Health had approved the procedures (decree n. 539/2021-PR). 8 BALB/c mice were randomized to two different diets: normal diet and EAArmd diet, following already published studies on lifespan [8]. Both diets (Dottori Piccioni s.r.l., Gessate, Milano-Italy) perfectly matched the same total macronutrients and micronutrients contents and provide same amounts of calories. Main difference was in type of nitrogen content, which was near 20% in weight, in both diets: EAA to non-EAA ratio (EAA/non-EAA) was <<0,9 in control diet, while EAArmd diet provided 84% of EAA, EAA/non-EAA <5). Small differences in nitrogen content evaluated at the end of production were linked to technical reasons in preparation of pellets and dehydration necessary to long term conservation. While normal diet respected strictly AIN76-A/NIH7 requirements, EAArmd (Nutrixam, Named S.r.i. Lesmo, Italy) provided nitrogen as free AA according the formulation presented in Table 2.

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Table 2. Composition (qualitive and quantitative) of macronutrients in pellets fed to the 2 groups of mice transfected with cancer cells. * Nitrogen (%) from free AAs only. ° Nitrogen (%) from vegetable and animal proteins and added AA. StD = Standard diet; EAArmd = Essential-AA rich modified diet; N = nitrogen. The dotted line represents the limit between EAA (upside) and non-EAA (beneath). L-Cystine was included to match Sulphur AA needs while minimizing methionine content [9]. bcaa = branched chain AA.

	StD	**EAArmd**
KCal/Kg	3952	3995
Carbohydrates (%)	54.61	61.76
Lipids (%)	7.5	6.12
Nitrogen (%)	21.8 °	20 *
Proteins: % of total N content	95.93	--
Free AA: % of total N content	4.07	100
EAA/non-EAA (% in grams)	-	86/14
Free AA composition (%)		
L-Leucine (bcaa)	--	13.53
L-Isoleucine (bcaa)	--	9.65
L-Valine (bcaa)	--	9.65
L-Lysine	0.97	11.6
L-Threonine	--	8.7
L-Histidine	--	11.6
L-Phenylalanine	--	7.73
L-Methionine	0.45	4.35
L-Tyrosine	--	5.80
L-Triptophan	0.28	3.38
L-Cystine	0.39	8.20
L-Cysteine	--	--
L-Alanine	--	--
L-Glycine	0.88	--
L- Arginine	1.1	--
L-Proline	--	--
L-Glutamine	--	--
L-Serine	--	2.42
L-Glutamic Acid	--	--
L-Asparagine	--	--
L-Aspartic Acid	--	--
Ornithine-αKG	--	2.42
N-acetyl-cysteine	--	0.97

After15 days of nutrition randomly assigned to mice, $1x10^5$ CT26 cells, ATCC code CRL-2638™ strain Balb/c, suspended in 100µl of physiological solution were subcutaneously injected in the same position (right hip) in a group of 8 animals, 4 controls and 4 EAArmd fed. After 21 days animals were sacrificed and tumors accurately isolated, measured (latitude, length, depth) and weighted. Autopsy was performed in any animal and organs (skeletal muscle, adipose tissue, heart, kidneys, liver….) sampled and stored appropriately.

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5. Conclusions

A main target of altered EAA/non-EAA is protein synthesis and autophagy balance. As we discussed elsewhere, protein synthesis and autophagy are not in opposition, but they work in a synchrony whose rhythms and intensities are dictated by ATP availability: the more ATP, the more would be implemented synthesis of proteins, which, being extremely expensive in terms of energy, would produce AMP in amounts proportional to the starting ATP concentration. The rise of AMP, in turn, would activate AMPK, which would blunt protein synthesis and activate autophagy allowing to restore ATP reserves and preventing mortal energy defaults [1].

Thus, both the balance among activation/inactivation of the upstream and downstream components of mTORCs and AMPK pathways should be examined in the attempt to understand *a)* which pathway is predominant in normally fed cancer cells, and *b)* what epigenetic level makes EAArmd fed cancer cells metabolically fragile.

We are interested in observing whether EAArmd diet increases frequency and/or amplitude of phases of AMPK driven autophagy, and/or whether some component of mTORCs is peculiarly regulated by altered EAA/non-EAA ratio. BCL2 anti- and pro-autophagy branches should also be studied, with a focus on BH3 only BIM/BID and BAD/BAX dependent paths [10]. Furthermore, calpains [11] should be investigated as likely involved as epigenetic drivers of the observed differences in growth. Of interest, membrane lipid synthesis inhibition triggered by AMPK, may be modulated by EAArmd: peculiarly PLD 1 and 2 [12] would be a main focus of our studies. Feeding cancer bearing patients with very high doses of EAA may unveil specific frailties of cancer cells, opening new options for cancer treatments.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Ethics Committee of University of Brescia, Animal Welfare Committee (OPBA) and by the Italian Ministry of Health, authorization number 539/2021-PR.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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