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Diminution of the development of experimental metastases produced by murine metastatic lines in essential fatty acid-deficient host mice

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In a previous study we found that the capacity for spontaneous metastases of tumors developed after subcutaneous transplantation of RSV-transformed Balb/c 3T3 cells was reduced in essential fatty acids (EFA)-deficient host animals. In the present study, we have extended our investigation by considering the requirement of EFA for the formation of lung colonies obtained by i.v. injection of two metastatic murine cell lines of different origin: (1) T3 cells, a highly metastatic cell line isolated from a fibrosarcoma, and (2) the F10 variant of B16 melanoma (B16-F10 cells). We found that EFA deficiency reduces the lung colonization of both T3 cells and B16-F10 cells without affecting the retention of tumor cells in the lung. NK cells did not seem to be involved in the diminution of lung colonization in EFA-deficient animals. Furthermore, by examining histologically the lung parenchyma at successive intervals after tumor cell injection, we found that, in comparison with control mice, EFA-deficient animals had fewer lung colonies and a prevalence of smaller microcolonies during the entire period of observation. This led us to conclude that the diminution in development of tumor colonies in the lungs of EFA-deficient host animals was related to a reduced growth rate of tumor cells at this site.

Keywords: B16 melanoma cells, essential fatty acid deficiency, experimental metastases, microscopic tumor colonies, T3 fibrosarcoma metastatic line

Introduction

According to a growing body of research, tumor progression and metastasis are influenced by dietary lipids [1–4]. In particular, diets enriched with linoleic acid have been shown to stimulate the formation of spontaneous metastases produced by the transplantation of human [5–7] and murine [8–11] mammary carcinoma lines. The strategy of these investigations has been to discover whether high levels of dietary linoleic acid promote the metastatic process.

In a previous study in our laboratory we found that essential fatty acid (EFA) deficiency reduced the capacity for spontaneous metastases from tumors developed after s.c. transplantation of RSV-transformed Balb/c 3T3 cells [12]. In that study, we used EFA deficiency as a way of determining whether the linoleic acid present in the tissues of a host maintained under normal dietetic conditions was required for tumor cells to metastasize to secondary organs. A requirement of EFA for the development of spontaneous metastases from a murine mammary carcinoma was reported by Eynard *et al*. [13].

We have recently extended this investigation on the requirement of linoleic acid for metastatic dissemination by considering models of experimental metastases, i.e. colonies reproduced in the host's organs after i.v. injection of tumor cells [14,15]. This assay represents a useful tool for exploring arrest and colonization steps in the metastatic cascade [14,15]. Therefore, we explored

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whether the formation of lung colonies was reduced in EFA-deficient animals injected with T3 cells, a line isolated from a murine fibrosarcoma, or with the F10 variant of the B16 murine melanoma (B16-F10 cells). In particular, we examined whether a diminution of lung colonies induced by EFA deficiency might be related to either reduced arrest and survival of tumor cells or reduced tumor cell growth in the secondary organs. Moreover, in view of the observation that the activity of NK cells is inhibited by PGE_2 [16,17], we considered the possibility that NK cells, an important effector of host defence [18,19] might be involved in the reduction of tumor cell lung colonization in host animals depleted of EFA, which are prostaglandin precursors.

Materials and methods

Diets

We have used a standard diet recommended for mice [20], composed of 70% sucrose, 16% casein, 3% α-cellulose, 1% choline chloride, 1% vitamins, 4% salt mixture, and 5% corn oil. The linoleic acid content of this diet was 1.8% (w/w). We have used an isocaloric EFA-deficient diet in which the corn oil was replaced with 3% hydrogenated coconut oil. Diets were purchased from Dottori Piccioni (Milano, Italy), and stored at 4°C in sealed plastic bags *in vacuo*.

Animals and dietary treatment

Newly weaned female mice (CBA and C57Bl/6 strains) were obtained from our animal facilities or from Charles River Italia (Calco, Italy). Ten to 40 mice per experiment were placed on the standard diet for one week and then divided into two groups: one group continued to be fed the standard diet (control animals), while the other group was switched to the EFA-deficient diet (EFA-deficient animals). From the beginning of the dietetic treatment, animals were weighed every week, and food consumption was assessed twice a week. The degree of EFA deficiency was determined by measuring the 20:3ω9/20:4ω6 ratio in liver phosphatidylcholine [21]. In CBA and C57Bl/6 mice fed the EFA-deficient diet for 5 weeks, this ratio was 2.1 ± 0.5 and 2.3 ± 0.9, respectively. EFA-deficient animals did not manifest, however, any significant decrease of growth rate or dermatologic symptoms (hair loss or scaly dermatitis) [22].

Cell lines and culture conditions

T3 cells and B16-F10 cells were kindly donated by Dr R. Bomford (Wellcome, Beckenham, UK) and Dr I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX), respectively. T3 cells were grown in Minimal Essential Medium (MEM) containing 1000 mg/l glucose (GIBCO, Life Technologies, MD) supplemented with 10% fetal calf serum (FCS) (Boehringer Mannheim, Germany) in a 5% CO_2 humidified atmosphere. B16-F10 cells were grown in Dulbecco's modified Eagle medium containing 4500 mg/l glucose (GIBCO) supplemented with 10% FCS in a 10% CO_2 humidified atmosphere. T3 cells (2.5×10^5) and B16-F10 cells (5.0×10^5) were seeded in 100 mm Falcon dishes and grown in 10 ml of their respective media. Cells were propagated every 3 days by incubation for 1 min with a 0.25% trypsin solution (GIBCO).

In experiments of NK cell depletion, we used a clone from the B16-F10 cell line (F10-M3 cells) donated by Dr S. Gattoni-Celli (Medical University of South Carolina, Charleston, SC). This clone was reported to produce metastases in the lung as well as the liver of triple immunodeficient animals [23]. F10-M3 cells were cultivated and propagated under the same conditions as those used for the parental B16-F10 cell line.

All cultures were periodically monitored for mycoplasma contamination by using Chen's fluorochrome test.

Depletion of NK cell activity

C57Bl/6 mice were depleted of NK cell activity by an i.p. injection of cyclophosphamide (240 mg/kg body weight) 4 days before being used for lung colonization assays [18].

Lung and liver colonization assays

Subconfluent cultures of T3 and B16-F10 cells were harvested by trypsinization, centrifuged at 200 ***g*** and then resuspended in PBS. Fifty thousand T3 cells or 1×10^5 B16-F10 cells in 0.2 ml of PBS were injected into the lateral tail veins of syngeneic CBA and C57Bl/6 mice, respectively, both fed a control or an EFA-deficient diet. Animals continued to be fed their respective diets until the end of the experiment. CBA and C57Bl/6 mice were sacrificed after 14 and 21 days, respectively, and lung surface colonies were counted with the aid of a dissecting microscope.

One hundred thousand F10-M3 cells were injected into control and EFA-deficient C57Bl/6 mice depleted of NK cells. After 21 days, lung and liver colonies were counted with the aid of a dissecting microscope. Lungs were also weighed in order to evaluate the total mass of tumor nodules, while liver nodules were measured with calipers.

Retention of tumor cells in the lung

Tumor cells were isotopically labeled by growth in a medium supplemented with 1μCi/ml of [^{3}H]choline (specific activity: 82 Ci/mmol) (Amersham International, UK) for 48 h. This radiolabel was efficiently incorporated into the cellular phospholipids. The radiolabeled cells were harvested, resuspended in PBS, and intravenously injected into syngeneic mice as described above. Tumor cell inocula contained approximately 2.4–3.0 $\times$ 10^6 dpm / 10^6 cells. At intervals of 1, 6, 24, and 96 h after tumor cell injection, 4–6 mice of each group were sacrificed and lipids were extracted from lungs by using Bligh and Dyer's method [24]. The retention of tumor cells in the lung at the stated intervals was determined by the percentage of the lipid-associated radioactivity administered to the animals.

Development of tumor colonies in the lung

The development of tumors in the lungs of mice which had been injected i.v. with T3 or B16-F10 cells was studied: (a) by counting the lung surface colonies, and (b) by examining histologically the microscopic colonies in mice sacrificed at different intervals after tumor cell injection. Histological examination was performed in hematoxylin-eosin-stained tissue sections (5 μm thick longitudinal sections) taken at regular intervals (10 sections every 50 μm) across the entire left lung. Microscopic tumor colonies were measured with the aid of an ocular micrometer and grouped according to their size ($<$ 50, 50–200, and $>$ 200 μm in diameter).

Statistical analysis

The statistical significance of the differences between the growth of tumor cells in control and in EFA-deficient animals was determined by the use of the Mann–Whitney test. Differences between slopes reported in Figures 2 and 3 were evaluated by using the least squares method.

Results

Effect of EFA deficiency on the colonization of T3 and B16-F10 cells in the lung

T3 and B16-F10 cells injected into EFA-deficient mice produced fewer lung colonies compared to those found in control mice (Table 1).

Effect of EFA deficiency on the colonization of F10-M3 cells in the lungs and livers of NK-depleted mice

F10-M3 cells produced a much greater number of lung and liver colonies in cyclophosphamide-treated mice than in untreated mice, an effect in agreement with Fidler's studies [18]. The potentiation of lung and liver colonization caused by NK depletion was diminished by EFA deficiency, which also reduced the lung weight and size of liver colonies (Table 2).

Effect of EFA deficiency on tumor cell retention in the lung

One hour after i.v. injection, 43% of T3 cells and 55% of B16-F10 cells were retained in the lungs of control mice; both types of cells were exponentially cleared thereafter. Arrest and clearance of T3 and B16-F10 cells were unchanged in EFA-deficient mice (Figure 1).

Effect of EFA deficiency on the development of tumor colonies in the lungs of mice injected with T3 and B16-F10 cells

As shown in Figure 2A, colonies became detectable on the lung surface 5 days after i.v. injection of T3 cells into control mice; the number of colonies increased at a rapid rate thereafter. Figure 2C shows that microscopic tumors with a diameter ranging from $<$ 50 to 200 μm were already present in the lung parenchyma 3 days after tumor cell injection. A progressive prevalence of larger tumors (diameter $>$ 200 μm) was obtained during later periods of observation. Compared with control mice, EFA-deficient mice injected i.v. with T3 cells showed

Table 1. Effect of EFA deficiency on tumor colonies in lungs of syngeneic mice injected intravenously with T3 and B16-F10 cells

Organ colonization	T3 cells		B16-F10 cells	
	Control	EFA-def. mice	Control	EFA-def. mice
Incidence[a]	5/5	8/8	4/4	3/3
No. of lung colonies[b]	76 (62–88)	7 (1–29)[c]	182 (180–210)	34 (29–71)[d]

[a] Number of mice with lung colonies / number of injected mice.
[b] Median number and range (in parentheses) of lung colonies per animal.
[c] Significantly different from control mice at $P < 0.002$.
[d] Significantly different from control mice at $P < 0.05$.

Table 2. Effect of EFA deficiency on lung and liver colonies produced by B16-F10 cells (M3 clone) injected i.v. into NK-depleted C57Bl/6 mice

Organ colonization	Intact mice	NK-depleted mice[a]	
		Control mice	EFA-def. mice
		Lung	
Incidence[b]	5/5	6/6	6/6
No. of lung colonies[c]	13 (10–27)	> 400	250 (205–320)[f]
Organ weight[d]	0.17 ± 0.05	0.92 ± 0.05	0.33 ± 0.04[f]
		Liver	
Incidence[b]	0/5	6/6	3/6
No. of liver colonies[c]	–	8.5 (3–14)	0.5 (0–2)[f]
Nodule diameter[e]	–	2.0 ± 0.5	0.3 ± 0.4[g]

[a]Depletion of NK cells in control and EFA-deficient mice was obtained by treatment with cyclophosphamide (240 mg/kg body weight) 4 days before tumor cell injection.
[b]Number of mice with lung or liver colonies / number of injected mice.
[c]Median number and range (in parentheses) of colonies per animal.
[d]Weight of the lung per animal in g (mean ± SD).
[e]Diameter of liver nodules in mm (mean ± SD).
[f]Significantly different from control mice at $P < 0.002$.
[g]Significantly different from control mice at $P < 0.02$.

fewer lung colonies (Figure 2B) and a prevalence of small and intermediate sized microcolonies (diameter < 50 and 50–200 μm) during the entire period of observation (Figure 2D).

As shown in Figure 3A, the development of lung surface colonies in control mice injected with B16-F10 cells was slower than that found in control mice injected with T3 cells (slopes: 3.31 vs 8.61). All the microscopic tumors detected in the lung parenchyma at an early stage of colonization of B16- F10 cells measured 50 μm in diameter (Figure 3C). Later, there was a progressive accumulation of microscopic tumors with a larger diameter. In EFA-deficient animals injected with B16-F10 cells there was a reduction of lung surface colonies (Figure 3B) and a prevalence of the smallest sized tumours during the entire period of observation (Figure 3D).

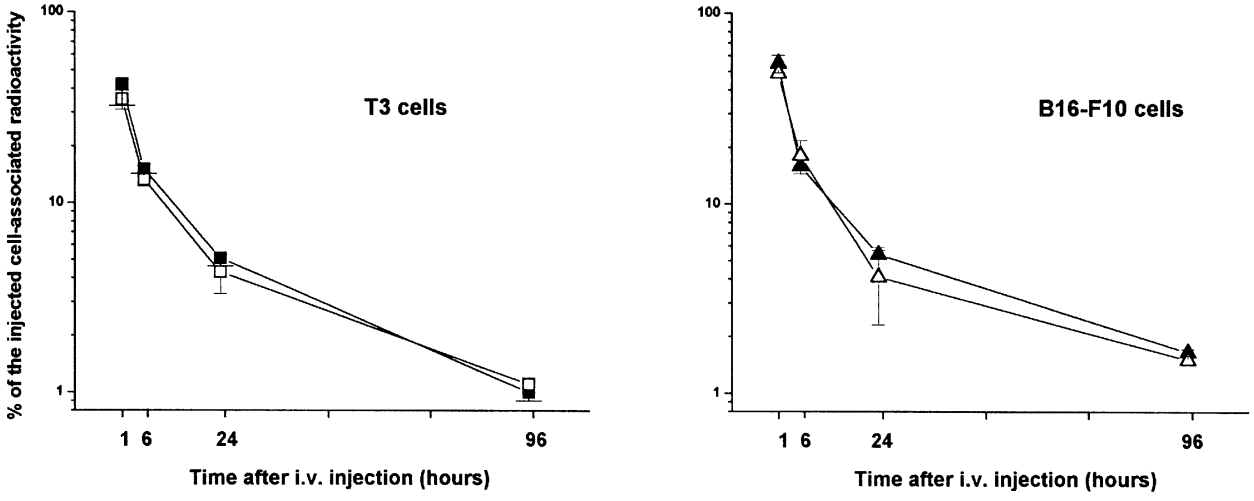

Figure 1. Retention of radiolabeled T3 and B16-F10 cells in the lungs of syngeneic control (solid symbols) or EFA-deficient mice (open symbols). Tumor cell retention was expressed on the basis of the lung-associated radioactivity measured at the stated intervals after i.v. injection of radiolabeled tumor cells. Each point represents the percentage (mean ± SEM of 4–6 mice) of cell-associated radioactivity injected into mice.

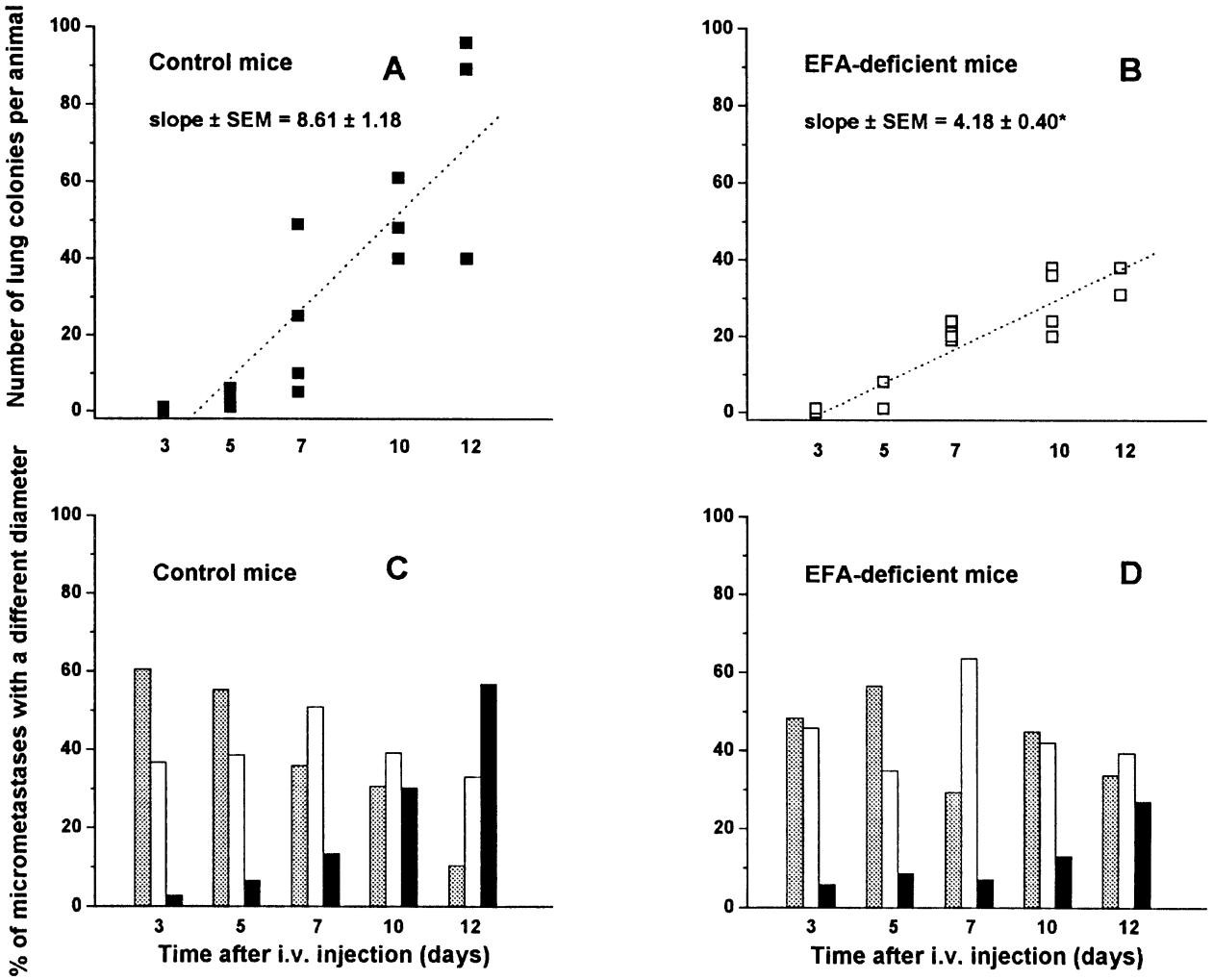

Figure 2. Effect of EFA deficiency on the development of lung colonies and microscopic tumors in mice injected with T3 cells. Lines in panels **A** (control mice) and **B** (EFA-deficient mice) were calculated from the linear regression analysis of lung colonies counted at the stated intervals after tumor cell injection. *Significantly different from control mice at $P < 0.01$. Panels **C** (control mice) and **D** (EFA-deficient mice) give the percent distribution of microscopic tumors grouped according to the diameter (< 50 μm: grey columns; 50–200 μm: white columns and > 200 μm: black columns) at the stated intervals after tumor cell injection.

Discussion

Previous studies in our laboratory had shown that the spontaneous metastatic capacity of RSV-transformed Balb/c 3T3 cells transplanted into EFA-deficient mice was reduced [12]. In the present study, we investigated whether EFA are important in the formation of experimental metastases (lung colonies) produced by two murine lines of different origin, the T3 fibrosarcoma and B16-F10 melanoma lines. This assay explores the mechanisms of arrest and lung colonization of tumor cells without the interference due to the unpredictable release of cells from the primary tumor. We found that T3 and B16-F10 cells injected into EFA-deficient mice had a reduced capacity for producing lung colonies, an effect not apparently related to a change in arrest and survival of tumor cells in the lung.

The formation of lung colonies might have been inhibited in EFA-deficient animals as a result of the potentiation of the NK cell activity, the reasoning being that the PGE_2-mediated suppression of NK cell activity [16,17] would have been relieved by the reduced biosynthesis of PGE_2 in EFA-deficient animals [25–27]. To clarify this point, we used an experimental protocol based on the treatment of EFA-deficient animals with cyclophosphamide, a well known inhibitor of NK activity [18]. If NK cells

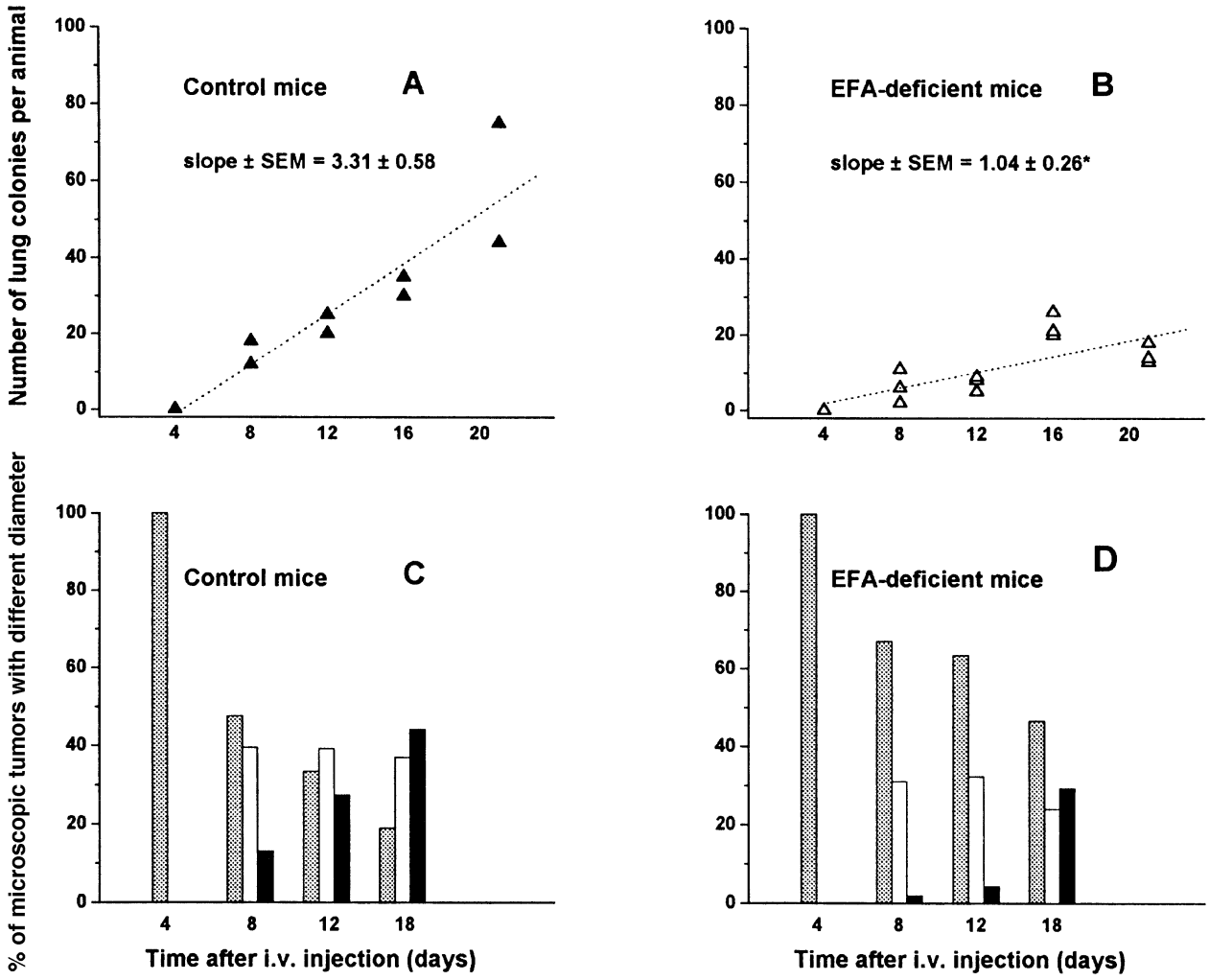

Figure 3. Effect of EFA deficiency on the development of lung colonies and microscopic tumors in mice injected with B16-F10 cells. Lines in panels (A) and (B) were calculated as reported in Figure 2. *Significantly different from control mice at $P < 0.01$. (C) and (D) as Figure 2.

were involved in the effect of EFA deficiency on tumor cell lung colonization, we would expect to find a greater lung colonization in EFA-deficient *vs* intact animals after NK depletion, analogous to that observed in NK-depleted animals fed a standard diet. However, we found that melanoma cells injected into NK-depleted EFA-deficient mice produced fewer lung colonies than in NK-depleted mice fed a standard diet. These data suggest that NK cells are not involved in the inhibitory effect of EFA deficiency on the lung colonization of tumor cells, although their function was not actually measured in this instance.

Compared with controls, EFA-deficient mice showed a slower development of tumor foci into detectable lung colonies, suggesting an inhibitory effect of EFA deficiency on the growth rate of the tumor cells. The requirement of EFA for growth of tumor cells is sustained by the growth-promoting activity of linoleic acid as reported by *in vitro* and *in vivo* studies [28,29]. In specific cell systems, a lipoxygenase-derived metabolite of linoleic acid, 13-HODE [30], modulates the epidermal growth factor signaling pathway leading to cell proliferation [31]. Moreover, crucial steps in growth control, such as activation of proto-oncogenes [32] and protein kinase C activity [33], were shown to be influenced by cyclo-oxygenase and lipoxygenase metabolites of EFA. The possibility that the requirement of EFA for tumor cell colonization is mediated by cyclo-oxygenase metabolites is also suggested by the observation that indomethacin inhibited the metastasis-promoting activity of linoleic acid [34].

In conclusion, our results demonstrate that EFA contained in the host's tissues promote growth of tumor cells in the lung. Moreover, in view of the regulatory role of PUFA on gene expression [35], EFA might represent one of the several factors of host organ microenvironment which modulate tumor cell gene expression and metastatic phenotype [36].

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References

1. Reddy BS, Cohen LA, McCoy EL *et al*. 1980, Nutrition and its relationship to cancer. *Adv Cancer Res*, **32**, 238–54.
2. Kollmorgen GM, King MM, Kosanke SD *et al*. 1983, Influence of dietary fat and indomethacin on the growth of transplantable mammary tumors in rats. *Cancer Res*, **43**, 4714–19.
3. Ip C, Carter CA and Ip MM, 1985, Requirement of essential fatty acid for mammary tumorigenesis in the rat. *Cancer Res*, **45**, 1997–2001.
4. Carrol KK, 1992, Dietary fat and breast cancer. *Lipids*, **27**, 793–97.
5. Rose DP, Connolly JM and Meschter CL, 1991, Effect of dietary fat on human breast cancer growth and lung metastasis in nude mice. *J Natl Cancer Inst*, **83**, 1491–94.
6. Rose DP, Hatala MA, Connolly JM *et al*. 1993, Effect of diets containing different levels of linoleic acid on human breast cancer growth and lung metastasis in nude mice. *Cancer Res*, **53**, 4686–90.
7. Rose DP, Connolly JM, and Liu X-H, 1994, Effects of linoleic acid on the growth and metastasis of two human breast cancer cell lines in nude mice and the invasive capacity of these cell lines *in vitro*. *Cancer Res*, **54**, 6557–62.
8. Hubbard NE and Erikson KL, 1987, Enhancement of metastasis from a transplantable mouse mammary tumor by dietary linoleic acid. *Cancer Res*, **47**, 6171–75.
9. Hubbard NE and Erikson KL, 1989, Effect of dietary linoleic acid level on lodgement, proliferation and survival of mammary tumor metastases. *Cancer Lett*, **44**, 117–25.
10. Katz EB and Boylan ES, 1987, Stimulatory effect of high polyunsaturated fat diet on lung metastasis from the 13762 mammary adenocarcinoma in female retired breeder rats. *J Natl Cancer Inst*, **79**, 351–58.
11. Katz EB and Boylan ES, 1989, Effect of the quality of dietary fat on tumor growth and metastasis from a rat mammary adenocarcinoma. *Nutr Cancer*, **12**, 343–50.
12. Calorini L and Ruggieri S, 1992, Reduction of the metastatic potential of a RSV-transformed fibroblastic line (B77-AA6 cells) upon transplantation in essential fatty acid-deficient mice. *Inv Metastasis*, **12**, 233–40.
13. Eynard AR, Quiroga P and Silva R, 1989, Dietary fatty acid composition modulates the metastatic behaviour of a mammary gland adenocarcinoma. *Cell Biol Int Reports*, **13**, 813–14.
14. Nicolson GL and Poste G, 1983, Tumor implantation and invasion at metastatic sites. *Int Rev Exp Pathol*, **25**, 77–181.
15. Hagmar B, Ryd W and Erkell LJ, 1983, Methods in metastasis research. *Inv Metastasis*, **3**, 1–21.
16. Young MRI, 1994, Eicosanoids and the immunology of cancer. *Cancer Metastasis Rev*, **13**, 337–48.
17. Lala PK and Saarloos MN, 1994, Prostaglandins and the host immune system: applications of prostaglandin inhibitors for cancer immunotherapy. In: Harris JE, Braun DP, Anderson K, eds. *Prostaglandin Inhibitors in Tumor Immunology*, CRC Press, pp. 187–227.
18. Hanna N and Fidler IJ, 1980, Role of natural killer cells in the destruction of circulating tumor emboli. *J Natl Cancer Inst*, **65**, 801–09.
19. Gorelik E, Wiltrout RH, Okumura K, *et al*. 1982, Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int J Cancer*, **30**, 107–12.
20. Bieri JG, Stoewsand GS, Briggs GM, *et al*. 1977, Report of the American Institute of Nutrition. *Ad hoc* committee on standards for nutritional studies. *J Nutr*, **107**, 1340–48.
21. Holman RT, 1960, The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. *J Nutr*, **70**, 405–10.
22. Holman RT, 1968, Essential fatty acid deficiency. In: Holman RT, ed. *Progress in the Chemistry of Fats and Other Lipids*, Oxford: Pergamon Press, pp. 275–348.
23. Gattoni-Celli S, Calorini L, Simile MM, *et al*., 1993, Modulation by MHC Class I antigens of the biology of melanoma cells. Non-immunological mechanisms. *Melanoma Res*, **3**, 285–89.
24. Bligh EG and Dyer WJ, 1959, A rapid method of total lipid extraction and purification. *Can J Biochem*, **37**, 911–17.
25. Croft KD, Beilin LJ, and Vandongen R, 1984, Dietary modification of fatty acid and prostaglandin synthesis in the rat. *Biochim Biophys Acta*, **795**, 196–207.
26. Mathias M and Dupont J, 1979, The relationship of dietary fats to prostaglandin biosynthesis. *Lipids*, **14**, 247–52.
27. Parnham MJ, Vincent JE, Zijlstra FJ, *et al*. 1978, The use of essential fatty acid deficient rats to study pathophysiological roles of prostaglandins. Comparison of prostaglandin production with some parameters of deficiency. *Lipids*, **14**, 407–12.
28. Holley RW, Baldwin JH and Kiernan JA, 1974, Control of growth of a tumor cell by linoleic acid. *Proc Natl Acad Sci USA*, **71**, 3976–78.
29. Rose DP and Connolly JM, 1989, Stimulation of growth of human breast cancer cell lines in culture by linoleic acid. *Biochem Biophys Res Commun*, **164**, 277–83.
30. Sobermann RJ, Harper TW, Betteridge D, *et al*. 1985, Characterization and separation of the arachidonic acid 5-lipoxygenase and linoleic acid ω-6 lipoxygenase

(arachidonic acid-15 lipoxygenase) of human polymorphonuclear leukocytes. *J Biol Chem*, **260**, 4508–15.
31. Eling TE and Glasgow WC, 1994, Cellular proliferation and lipid metabolism: importance of lipoxygenases in modulating epidermal growth factor-dependent mitogenesis. *Cancer Metastasis Rev*, **13**, 397–410.
32. Handler JA, Danilowicz RM, and Eling TE, 1990, Mitogenic signaling by epidermal growth factor (EGF), but not platelet-derived growth factor, requires arachidonic acid metabolism in Balb/c 3T3 cells. *J Biol Chem*, **265**, 3669–73.
33. Honn KV, Tang DG, Gao X, *et al*. 1994, 12-Lipoxygenases and 12 (S)-HETE: role in cancer metastasis. *Cancer Metastasis Rev*, **13**, 365–96.
34. Hubbard NE, Chapkin RS and Erikson KL, 1988, Inhibition of growth and linoleate-enhanced metastasis of a transplantable mouse mammary tumor by indomethacin. *Cancer Lett*, **43** , 111–20.
35. Clarke SD and Jump DB, 1994, Dietary polyunsaturated fatty acid regulation of gene transcription. *Ann Rev Nutr*, **14**, 83–98.
36. Radinsky R, 1995, Modulation of tumor cell gene expression and phenotype by the organ-specific metastatic environment. *Cancer Metastasis Rev*, **14**, 323–38.