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REVIEWS: CURRENT TOPICS

Dietary factors and growth and metabolism in experimental tumors

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Abstract

Development of a diet that provides adequate nutrition and effective cancer prevention is an important goal in nutrition and cancer research. A confounding aspect of dietary control of tumor growth is the fact that some nutrients may up-regulate tumor growth, whereas other nutrients and nonnutrients down-regulate growth. Both up- and down-regulators may be present in the same foodstuff. Identification of these substances, determination of their mechanisms of action and potencies, as well as the interactions among the different mechanisms are topics of ongoing research. In this review, we describe results obtained in vivo or during perfusion in situ using solid tissue-isolated rodent tumors and human cancer xenografts in nude rats. Linoleic acid (LA), an essential n-6 polyunsaturated fatty acid (PUFA), was identified as an agent in dietary fat that is responsible for an up-regulation of tumor growth in vivo. Tumor LA uptake, mediated by high intratumor cAMP, stimulated formation of the mitogen, 13-hydroxyoctadecadienoic acid (13-HODE) and also increased ERK1/2 phosphorylation, [^{3}H]thymidine incorporation and growth. A mechanism for control of this growth-promoting pathway was revealed during studies of the effects of dietary nutrients and nonnutrients known to inhibit tumor growth. These included four groups of lipophilic agents: n-3 fatty acids, melatonin, conjugated LA isomers and trans fatty acids. Each of these agents activated an inhibitory G protein-coupled receptor-mediated pathway that specifically suppressed tumor uptake of saturated, monounsaturated and n-6 PUFAs, thereby inhibiting an early step in the LA-dependent growth-promoting pathway.
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1. Introduction

Consumption of diets that are adequate for energy, but low in red meat and fat and enriched in fish, vegetables, fruit, nuts and herbs, has been recommended as an important and positive way to decrease the risk of cancer in the world [1,2]. It was suggested that cancer incidence could feasibly be reduced by as much as 30% to 40% by properly selected dietary changes [1]. These dietary guidelines were drawn from a large number of epidemiological studies indicating that ingestion of red meat, animal fats and some plant-derived oils increased cancer incidence [1–3]. In contrast, ingestion of vegetables, fruit, plant-derived oils such as olive and flaxseed oils, and marine fish and their oils was associated with a reduction in cancer risk [1–3]. Many nutritional studies in experimental animals confirmed both the cancer-promoting effects of animal fats and certain vegetable oils [1–3] and the protective effects of marine fish oils [3] and the many nonnutritive components present in fruits, vegetables, nuts and herbs and spices [4]. Recommendations were that total fat intake should range from 15% to not more than 30% of total energy needs and should include oils from marine fish when possible [1–3]. Although cause-and-effect associations derived from epidemiological and nutritional studies in experimental animals may be strong and convincing, evidence for control of tumor growth and metabolism by nutrients and nonnutrients is further strengthened if a mechanism is identified and shown to be biologically reasonable [1–3]. Thus, for those dietary agents that are known to influence growth of solid tumors in vivo or tumor cell lines in vitro there is a great interest in

Abbreviations: 8-Br-cAMP, 8-bromo-cyclic adenosine monophosphate; CLA, conjugated linoleic acid; COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; EFA, essential fatty acid; EFAD, essential fatty acid-deficient; EPA, eicosapentaenoic acid; ER, estrogen receptor; FATP, fatty acid transport protein; GPCRs, G protein-coupled receptors; LCACS, long-chain acyl-CoA synthetase; pAkt, phosphorylated-amino kinase terminal kinase; pERK1/2, phosphorylated extracellular signal-regulated kinase1/2; pMEK, phosphorylated-mitogen activated protein kinase; FA, fatty acid; 13-HODE, 13-hydroxyoctadecadienoic acid; LA, linoleic acid; PTX, pertussis toxin; PUFA, polyunsaturated fatty acid.

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determining the mechanism of their actions and their potential interactions; such information might apply to human tumors and the human diet.

In this review, we summarize experiments in which the uptake and metabolism of the major nutrients in host plasma were measured in solid rodent and human cancer xenografts in nude rats studied either in vivo or during perfusion in situ. Tumors actively consumed all major nutrients in host blood plasma, but the positive rate of tumor growth was dependent primarily on the linoleic acid (LA) content of host plasma lipids. Mechanistic information on the role of LA in tumor growth promotion and on four dietary components known to attenuate growth of solid tumors is described. These four agents are as follows: three macronutrients [n-3 polyunsaturated fatty acids (PUFAs), conjugated LA (CLA) isomers and trans fatty acids (FAs)] and the nonnutrient melatonin. The review concentrates on possible mechanisms that operate in vivo and during initial interactions among these five dietary factors in transplantable solid tumors during perfusion in situ. Results of studies by other investigators who have used tissue-isolated rodent and human cancer xenografts in vivo or during perfusion in situ were included. Research into the effects of n-6 and/or n-3 PUFAs and of other nutrient factors on growth and metabolism in rodent and human cancer cells lines in vitro has also developed a large literature. Pertinent in vitro studies that provided added insights into the proposed mechanisms were included.

Although complex and technically difficult to study in vivo or during perfusion in situ, solid tumors are, we believe, the best way to measure tumor–host relationships influenced by dietary and/or host-derived substances. Solid tumors necessarily include nontumor cells, e.g., vascular tissues and stromal cells, which may contribute substantially to the metabolism and growth properties of the tumor [5,6]. Vascular tissues and stromal cells that populate human tumors in vivo are different from those that populate human cancer xenografts in nude rats. The effect that nude rat stromal cells may have on metabolism and growth of human cancer xenografts is unknown, but positive growth effects are possible [5]. It is worth noting that vascular and stromal cells are not present in experiments performed with either rodent or human cancer cell lines in vitro.

2. Tumor nutrient uptake and metabolism

2.1. Glucose and lactate

Fast-growing, solid rodent tumors utilize large amounts of the nutrients contained in host arterial blood for growth and energy. Arteriovenous blood difference measurements across tissue-isolated rat tumors in vivo and during perfusion in situ [7–9] indicated that the glucose concentration in arterial blood plasma in rats bearing four different tumors ranged from 4.4 to 10.8 mM (7.4±0.4 mM, mean±S.D.). Rates of glucose supply, which depended on the arterial plasma concentration and blood flow rate, ranged from 200 to 1200 nmol glucose/min per gram tumor. Despite this large range in supply rates, all tumors utilized about 25% of the plasma glucose during one pass of arterial blood through the tumor. Arterial blood plasma glucose concentrations in tumor-bearing rats fasted for 2 days were lower (6.6±1.4 mM, mean±S.D.) [9] than values observed in fed rats [8]. However, tumor glucose uptake was increased in fasted rats; 30% to 50% of the glucose supplied was utilized [9]. Regression analyses of tumor glucose utilization vs. supply rates in fed or fasted rats indicated that the slopes were significantly different ($P<.05$), suggesting that fasting may have increased the efficiency of glucose uptake. There was no evidence that the rate of glucose uptake in vivo reached a maximum in tumors in either fed or fasted host rats [8,9] or in human breast cancer xenografts [10,11] in fed nude rats.

All tumors released lactate when the arterial blood concentration was less than 2 to 3 mM. Surprisingly, when the arterial blood plasma lactate concentrations were above 2–3 mM lactate was utilized as a carbon source [8,9,12–14]. Thus, both glucose and lactate may be utilized at the same time. The data suggested that glucose utilization depended on the glucose supply rate but that tumor lactate production or utilization resulted from an equilibration between the variable arterial lactate concentration and the more constant intratumor lactate concentration [12]. If the arterial lactate concentration was less than, greater than, or equal to the intratumor lactate concentration, the tumor, respectively, produced, utilized, or neither produced nor utilized lactate [12]. There was no evidence that tumor growth rates were dependent on arterial blood plasma levels of either glucose or lactate in fed or fasted rats.

2.2. Ketone bodies

Arterial blood ketone body concentrations were low (0.15–0.18 mM) in fed tumor-bearing rats [9]. However, acetoacetate and β-hydroxybutyrate levels in arterial blood plasma were increased to 0.72 and 3.0 mM, respectively, in rats subjected to an acute fast [9]. Similar arterial blood plasma ketone body concentrations were observed in tumor-bearing rats following onset of streptozotocin-induced diabetes [15]. In diabetic rats, tumor ketone body utilization was directly proportional to the rate of supply in the arterial blood plasma [15]. Rates of utilization for acetoacetate and β-hydroxybutyrate were 14 and 25 nmol/min/g, respectively, and represented about 30% of the supply rate [9]. The initial enzyme required for acetoacetate utilization in peripheral tissues, succinyl-CoA:acetoacetyl-CoA transferase, though absent from normal liver, is present in rat hepatomas and is increased in activity in proportion to hepatoma growth rate [16]. Ketone bodies were also utilized by human breast cancer xenografts [10,11] and by human squamous cell head and neck carcinomas in vivo [17]. Ketone body utilization in human tumors appeared to be linearly related to the supply.

Evidence suggested that ketone body carbon is utilized for both energy and growth.

2.3. Amino acids

Amino acids were actively removed from the arterial blood plasma by four different tissue-isolated rat tumors. Examination of these rat tumors revealed that 10% to 20% of the amino acids were removed during one pass of the arterial blood [8,9,13]. Uptake of glutamine, the most abundant amino acid, occurred at about 30% of the supply rate and was especially prominent in fasted rats. Regression analyses of glutamine utilization rates in fasted and fed rats indicated that uptake in fasted rats was significantly greater ($P<.05$) than in fed rats. An acute fast appeared to make the processes for glutamine and glucose utilization more efficient. All rat tumors released ammonia and two tumors released alanine and/or glycine [8,9] indicating the amino acids were actively oxidized and metabolized as well as incorporated into cellular proteins. Tumor growth in rats fed a nutritiously adequate diet was not limited by amino acid availability.

2.4. Fatty acids

Utilization of plasma FAs was observed in rat hepatomas 7288CTC [18–26] and 7777 [18] and in either estrogen receptor positive (ER^+) [27–29] or negative (ER^-) [27,30] MCF-7 human breast cancer xenografts. Tumor-bearing rats were fed laboratory chow (4.1% fat) or semipurified diets that contained 2% to 10% fat. Total FAs included the seven major FAs in rat arterial blood plasma: myristic, palmitic, palmitoleic, stearic, oleic, LA and arachidonic acids. Rat hepatomas removed total FAs from each of the four major plasma lipid classes: free FAs, triacylglycerols, phospholipids and cholesterol esters. Total FA uptake by rat hepatomas 7288CTC and 7777 was 30% to 50% and 50%, respectively, of the total FAs supplied in arterial blood. ER^+ MCF-7 human breast cancer xenografts removed about 13% to 16% of the FAs supplied, whereas ER^- MCF-7 xenografts removed about 22% of the FA supply. Rates of FA uptake in rat tumors were about doubled in tumor-bearing rats subjected to either an acute fast [31] or streptozotocin-induced diabetes [15]. The hyperlipemia that resulted from these treatments caused a 400% increase in the total FA content of host arterial blood plasma [15,32,33]. There was no evidence that total FA uptake was saturated at the high plasma lipid concentrations observed in fasted rats.

All major substrate groups in host arterial blood were actively taken up and metabolized, indicating that previous characterizations of tumors in vivo as "glucose traps" [34] and "nitrogen traps" [35] were appropriate. Tumor uptake of FAs from plasma lipids was an especially prominent feature. As judged from the efficiencies of uptake of glucose, lactate, amino acid ketone bodies and FAs, tumors in vivo might better be considered as "nutrient traps." Few nutrients, except for lactate, alanine and glycine, were returned to the host circulation. This property of avid substrate accumulation in solid tumors in vivo would contribute to a cachectic effect in the host.

3. Evidence for LA-dependent tumor growth

3.1. Role of dietary LA

The importance of dietary fat in experimental tumorigenesis [36] and growth regulation in established cancers [37] in experimental animals has been known for more than 60 years. Diets containing high corn oil contents selectively activated growth of transplantable rodent mammary tumors [38], rat hepatoma 7288CTC [21] and MDA-MB-435 human breast cancer xenografts [39]. Chemically induced carcinogenesis in the rat mammary gland [40], pancreas [41] and colon [42] was also increased by high dietary corn oil contents. About 65% of the FA content of corn oil is LA. Significant positive correlations were found among the corn oil content of the diet, serum LA content and the incidence of mammary carcinomas induced by *N*-nitromethylurea in female F344 rats [43]. Increased dietary corn oil was positively correlated with both increased plasma levels of LA and arachidonic acid, as well as growth of rat hepatoma 7288CTC in vivo [21]. Since host rat tissues convert LA to arachidonic acid, it was not possible to separate the effects on tumor growth of either FA in vivo in experimental animals fed an essential fatty acid (EFA)-sufficient diet. However, in tumor-bearing mice fed an EFA-deficient (EFAD) diet addition of either 0.1% or 0.5% purified arachidonic acid to the diet had no significant effect on tumor growth, whereas addition of 0.1% purified LA to the diet significantly increased tumor growth [44]. No correlations were observed between tumor growth and the plasma levels of either saturated or monounsaturated FAs [21]. Meta-analysis of 97 reports on the effects of different dietary FAs on mammary tumor incidence covering the years 1966 to 1995 concluded that n-6 essential FAs were responsible for the growth-enhancing effect of dietary fats [45]. Saturated FAs had a weak effect and monounsaturated FAs had no effect [45].

3.2. Tumor LA uptake

An acute fast [9,31], acute under-feeding [21] or streptozotocin-induced diabetes [15], metabolic conditions that caused hyperlipemia in the arterial blood of the tumor-bearing rat, increased growth and [^{3}H] thymidine incorporation by 70% to 400% in tissue-isolated rat hepatomas 7288CTC and 5123C and in Jensen sarcoma and Walker carcinoma 256. Experiments designed to distinguish between the relative growth-promoting effects of LA and arachidonic acid in hyperlipemic blood indicated that both FAs contributed, but that LA was more effective [33]. Measurements of the relative potencies of the tumor growth-enhancing effects of LA vs. arachidonic acid were performed using Buffalo rats fed an EFAD diet [19]. After 8 to 12 weeks on this diet, plasma LA was undetectable in

arterial blood plasma and the arachidonic acid concentration was 0.01 ± 0.1 mM. Hepatomas 7288CTC implanted in EFAD rats grew very slowly. Perfusion of these tumors in situ with donor EFAD arterial blood to which increasing plasma concentrations of either LA or arachidonic acid were added indicated that both LA and arachidonic acid increased tumor [^{3}H]thymidine incorporation. However, the plateaus in [^{3}H]thymidine incorporation observed for LA and arachidonic acid were 350 and 125 dpm/μg tumor DNA, respectively, indicating that LA was three to four times more effective than arachidonic acid in increasing tumor [^{3}H]thymidine incorporation [19]. Most interestingly, the growth-enhancing effects of LA and arachidonic acid were additive [19,33], suggesting that the two n-6 FAs activated different mechanisms for growth promotion.

3.3. 13-HODE formation

The rate of tumor release of 13-HODE into the venous blood was directly proportional to the rate of LA uptake [21]; 2% to 5% of the plasma LA taken up was converted to 13-HODE in rat hepatoma 7288CTC. Radiolabeled LA, introduced into the arterial blood during perfusion in situ, was converted to 13-HODE and 13-ketooctadecadienoic acid (13-KODE), a metabolite of 13-HODE [21]. Evidence indicated that 13-HODE formed from plasma LA was responsible for the growth-enhancing effect of LA [23]: (i) In rats fed a LA-replete diet, the addition of a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA, 10 μM in plasma), to the arterial blood during perfusion of hepatoma 7288CTC in situ abolished tumor release of 13-HODE, but did not affect tumor LA uptake. [^{3}H]Thymidine incorporation in NDGA-treated tumors was about 50 dpm/μg tumor DNA and was increased to 450 dpm/μg tumor DNA by addition of 13-HODE to the NDGA-containing arterial blood. Addition of either 13-KODE or 9-hydroxyoctadecadienoic (9-HODE) acid had no effect. (ii) [^{3}H]Thymidine incorporation in hepatoma 7288CTC in EFAD rats perfused in situ with donor blood from EFAD rats was about 20 dpm/μg tumor DNA. Addition of 13-HODE to the EFAD donor arterial blood plasma caused dose-dependent increases in tumor 13-HODE uptake and [^{3}H]thymidine incorporation (to 400 dpm/μg tumor DNA) [23].

3.4. Tumor LA uptake, 13-HODE release and growth

Positive correlations were observed in vivo among rates of LA uptake, 13-HODE release and growth in tissue-isolated rat hepatoma 7288CTC in Buffalo rats [46] and in human xenografts of ER$^+$ and ER$^-$ MCF-7 breast [27,29,30], PC3 prostate, CFDT1 renal transitional and FaDu pharyngeal carcinomas in nude rats (unpublished results). These relationships in tumors in rats fed an EFA-sufficient diet are shown in Fig. 1. Linoleic acid-dependent growth in each of these tumors was also characterized by increased levels of intratumor cAMP [25,27,29,30,46,47], phosphorylated-amino kinase terminal kinase (pAkt) (unpublished results), phosphorylated-mitogen activated protein kinase (pMEK) [48], phosphorylated extracellular signal-regulated kinase (pERK1/2) [25,27,29,30,47,48]

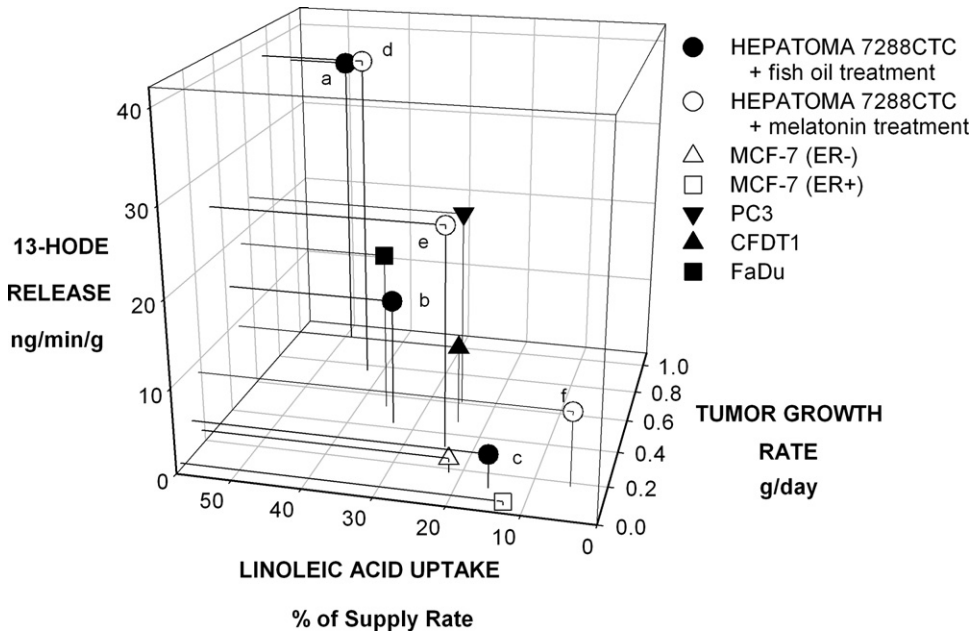

Fig. 1. A three-dimensional plot of the relationships among tumor LA uptake, 13-HODE release and growth rate in rat hepatoma 7288CTC and human cancer xenografts in vivo. All tumor-bearing rats were fed EFA-sufficient diets. Buffalo rats bearing hepatoma 7288CTC were treated as follows: (●) the diets contained (a) no fish oil, (b) 2% fish oil or (c) 4% fish oil; and (○) the rats ingested (d) no melatonin, (e) 0.5 μg melatonin/day or (f) 5 μg melatonin/day. The five human cancer xenografts are ER$^-$ (△) and ER$^+$ (□) MCF-7 breast; PC-3 prostate (▼); CFDT1 renal transitional (▲) and FaDu pharyngeal (■) carcinomas. Regression analysis of tumor growth against 13-HODE release (extensions from the data points to the left wall of the cube) demonstrated a significant positive correlation [R^2=0.775, P<.001, tumor growth=0.098+(0.02) (13-HODE release)]. Extensions from the data points to the base of the cube indicated a significant positive correlation between tumor growth and LA uptake [R^2=0.798, P<.001, tumor growth=−0.11+(0.02) (LA uptake)].

and [^{3}H]thymidine incorporation [21,25,27,30,33,46–48]. Fig. 1 also shows that the presence of either n-3 FAs or melatonin in the diets reduced LA uptake, 13-HODE release and growth in rat hepatoma 7288CTC in vivo. The inhibitions caused by these agents were dose dependent.

4. Dietary factors that suppress LA-dependent tumor growth

4.1. n-3 Fatty acids

The growth rates of transplantable rodent tumors [46,49–51] and human cancer xenografts in immunodeficient rodents [52,53] were decreased during the ingestion of dietary fish oil. Addition of purified n-3 FAs to the arterial blood supplying tissue-isolated solid tumors [19,29,49] suggested that n-3 FAs and not metabolites were responsible for the growth inhibition. Also, measurements of either [^{3}H]thymidine incorporation during perfusion in situ [19] or growth in vivo [46] suggested that the ratio of LA/n-3 FAs in host arterial blood plasma was important. However, definition of a mechanism for the n-3 FA-induced growth inhibition in solid tumors has been elusive. Two types of experiments were performed in an attempt to better understand the interactions that occurred between LA and n-3 FAs: (i) the chronic effects of dietary fish oil on growth and metabolism were examined in hepatoma 7288CTC in vivo and during perfusion in situ [46]; and (ii) the acute effects caused by addition of purified n-3 FAs to the arterial blood were examined on hepatoma 7288CTC [19,23] and on human cancer xenografts during perfusion in situ [29,30,47].

4.1.1. Dietary fish oil

Buffalo rats bearing tissue-isolated hepatoma 7288CTC were fed three LA-replete diets that contained 10% total fat; the control diet contained no fish oil and either 2% or 4% fish oil was present in the treatment diets [46]. Tumor-bearing rats fed the control or diets containing fish oil were exposed to constant light to suppress nocturnal melatonin secretion [28]. The LA contents of the three diets were not different. EPA, DHA and α-linolenic and stearidonic acids were the major n-3 FAs in the fish oil preparation. Tumor growth rates were measured throughout the treatment periods. When tumors weighed 4 to 6 g, arterial and tumor venous blood samples were collected and tumors were freeze-clamped for analyses. Rates of tumor FA uptake and 13-HODE release were calculated by arterio-venous difference measurements. The n-3 FA contents in the arterial blood collected from rats fed the control, 2% or 4% fish oil diets were 0, 76 and 134 μg/ml plasma, respectively. Relative to tumors in rats fed the control diet, tumors in rats fed either the 2% or 4% fish oil diets showed the following significant ($P<.05$) changes: (i) the intratumor cAMP contents were reduced 40% in each treatment group; (ii) rates of LA uptake and 13-HODE release (see also Fig. 1) were reduced 40% and 70%, respectively; (iii) [^{3}H]thymidine incorporation and DNA content were reduced 40% and 70%, respectively; and (iv) tumor growth rates were reduced from 1.2 g/day (control diet) to 0.4 and 0.1 g/day, respectively.

The relationships among the inhibitions of intratumor cAMP content, rates of FA uptake, 13-HODE release and [^{3}H]thymidine incorporation observed in tumors in rats fed the 2% fish oil diet were examined during perfusion in situ. Arterial blood for perfusion was collected from donor rats fed the 2% fish oil diet and exposed to constant light. Sequential arterial and tumor venous blood samples were collected across the tumors before and after treatment with either pertussis toxin (PTX) or 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP). Tumors were collected for analysis at the end of the perfusion. The results showed that PTX and 8-Br-cAMP completely reversed the inhibitions observed in rats fed 2% fish oil diet. All values for intratumor cAMP, rates of FA uptakes, 13-HODE release and [^{3}H]thymidine incorporation were returned to those observed in tumors in rats fed the control diet. Moreover, the reversal of the inhibitions occurred within 1–2 min after the donor blood containing either PTX or 8-Br-cAMP reached the tumor [46,47]. PTX catalyzes the ADP-ribosylation of the α subunit of inhibitory heterotrimeric guanine nucleotide G protein-coupled receptors (GPCRs) and reactivates the inhibited adenylyl cyclase activity [54]. 8-Br-cAMP is a cell permeable analog of cAMP that is resistant to hydrolysis by phosphodiesterases [55]. The results provided strong evidence that dietary n-3 FAs acted via an inhibitory GPCR to promote a dose-dependent inhibition of the signaling pathway required for LA-dependent tumor growth. Restoration of the complete pathway by either PTX or 8-Br-cAMP indicated that cAMP was required at an early step. The putative n-3 FA receptor has not yet been identified.

4.1.2. Purified n-3 FAs during perfusion in situ

Experiments performed during perfusion in situ provided additional information about the sequential steps in the LA-dependent signaling pathway in hepatoma 7288CTC [23] and in ER$^+$ [29] and ER$^-$ [47] MCF-7 human breast cancer xenografts. In these experiments, all rats, tumor-bearing and blood donors, were fed a LA-sufficient diet (no dietary n-3 FAs were present). Perfusions were performed in the morning when plasma melatonin concentrations were low [24]. Eicosapentaenoic acid added to the donor arterial blood during the perfusion in situ suppressed intratumor cAMP, FA uptake, 13-HODE release, phosphorylation of ERK1/2 and incorporation of [^{3}H]thymidine into tumor DNA. Each of these EPA-induced inhibitions was reversed by addition of either PTX or 8-Br-cAMP to the EPA-containing arterial blood. However, addition of 13-HODE to the EPA-containing arterial blood had no effect on the suppressed rate of FA uptake. Rather, the 13-HODE addition restored phosphorylation of ERK1/2 and [^{3}H]thymidine to control values; 13-HODE, formed from LA, was

the agent required for pERK1/2 phosphorylation, [^{3}H]thymidine incorporation and LA-dependent growth.

The effectiveness of n-3 FAs in arterial blood was determined in hepatoma 7288CTC during perfusion in situ [19]. Tumor-bearing and blood donor rats were fed an EFAD diet to deplete the arterial blood of essential FAs. Plasma concentrations of LA and n-3 FAs in donor blood were adjusted by addition of exogenous FAs. In the presence of 0.5 mM LA, addition of EPA, DHA, α-linoleic or stearidonic acid caused a dose-dependent reduction in FA uptake and [^{3}H]thymidine incorporation. The K_i values for α-linolenic acid and EPA inhibitions of total FA and LA uptake and [^{3}H]thymidine incorporation in hepatoma 7288CTC were 0.18 and 0.25 mM, respectively [19]. These K_i values were about midway between the arterial blood plasma n-3 FA concentrations measured in vivo in rats fed diets containing 2% or 4% fish oil [46].

4.2. Melatonin

Melatonin (*N*-acetyl, 5-methoxytryptamine) is the principal neurohormone of the pineal gland. It is derived from the amino acid tryptophan and is secreted into the venous blood in darkness. Nocturnal melatonin secretion is an important regulator of the circadian rhythm and plays a role in many physiological and pathophysiological functions, including carcinogenesis and growth regulation in transplanted solid tumors ([56], review). Melatonin (phytomelatonin) is also present in many edible plants [26,56,57]; nuts and seeds are good sources (1000 to 2000 pg/g dry tissue). The actions of melatonin on target tissues are believed to be mediated by the inhibitory melatonin GPCRs (MT_1 and MT_2). Melatonin in arterial blood plasma binds to these receptors and leads to the suppression of cAMP production in the target cells ([56], review). Rat hepatoma 7288CTC expresses both MT_1 and MT_2; MCF-7 human breast cancer xenografts express only functional MT_1 [27].

Melatonin is the most potent inhibitor of FA uptake in hepatoma 7288CTC and MCF-7 breast cancer xenografts yet discovered [24,26]. In Buffalo rats exposed to diurnal lighting (12L:12D), sufficient melatonin (60 pg/ml plasma) was secreted during the dark phase to completely inhibit FA uptake and 13-HODE release in hepatoma 7288CTC in vivo [24]. As the plasma melatonin concentration declined, tumor FA uptake increased and reached a value typical of the light phase at 0800 h [24]. A dose–response relationship was also observed in hepatoma 7288CTC during perfusion in situ. Donor blood for perfusion was collected from pinealectomized rats (no measurable melatonin) and increasing amounts of melatonin were added; a 50% inhibition of LA uptake, 13-HODE production and [^{3}H]thymidine incorporation was observed at a plasma melatonin concentration of 0.1 nM [26]. Perfusion of ER^+ and ER^- MCF-7 human breast cancer xenografts in situ with arterial blood containing 1 nM melatonin reduced LA uptake and 13-HODE release to zero, decreased pERK1/2 and caused 50% and 70% decreases in intratumor cAMP content and [^{3}H]thymidine incorporation, respectively [27]. Dietary supplementation with melatonin was tested on growth of hepatoma 7288CTC in pineal intact rats. Control rats were fed a 5% corn oil-melatonin-free diet, rats in the experimental groups were fed the control diet to which sufficient melatonin was added to provide a dose of either 0.5 or 5 μg/day. Tumors in rats that ingested dietary melatonin showed significant dose-dependent suppressions of FA uptake, 13-HODE release, [^{3}H]thymidine incorporation, DNA content and growth rate [26] (see Fig. 1).

The reactions of both hepatoma 7288CTC and MCF-7 human breast cancer xenografts to endogenous and dietary melatonin were reversed by addition of PTX, 8-Br-cAMP or forskolin to the melatonin-containing arterial blood [24,26]. Addition of 13-HODE to the melatonin-containing arterial blood reversed the inhibition of [^{3}H]thymidine incorporation but had no effect on FA uptake [24]. Addition of S20928, a specific melatonin GPCR antagonist, to the melatonin-containing donor blood completely suppressed the negative effects of melatonin on LA-dependent tumor growth [24,26], evidence that the changes induced by melatonin were receptor mediated.

4.3. Conjugated LA isomers, trans FAs and 9-HODE

Conjugated LA isomers and trans vaccenic acid are metabolites of LA formed in ruminants during incomplete bio-hydrogenation and isomerization. They are present in meat, milk fat, cheese and other food products derived from ruminants [58]. Ingestion of CLA isomers by experimental animals was shown to have several physiological effects ([59], review), including negative effects on carcinogenesis [60,61] and growth of human cancer xenografts in SCID mice [62]. Mechanisms of action of CLA isomers in experimental animals are of great interest and are under active investigation [59], but in humans it is unclear if the amounts of these agents ingested in a normal diet are functionally significant [63]. In addition, current dietary guidelines suggest that the content of meat and dairy products should be reduced [1,2]; these foodstuffs are a major dietary source of CLA isomers.

The trans FAs, elaidic and linoelaidic acids, are formed during the hydrogenation of vegetable oils to prepare margarines and vegetable shortening. 9-HODE is a hydroxylated CLA isomer formed in tissues during 15-lipoxygenase-1 activity [64]. The carbon skeleton of 9-HODE is identical to that of an active CLA isomer, t10,c12-CLA. Experiments performed using hepatoma 7288CTC and inguinal fat pads in Buffalo rats perfused in situ [25] provided evidence that addition to the arterial blood of certain CLA isomers, elaidic and linoelaidic acids, and 9-HODE inhibited tumor FA uptake, intratumor cAMP content, 13-HODE release, pERK1/2 and [^{3}H]thymidine incorporation into tumor DNA. Similar responses were observed in ER^- MCF-7 breast cancer xenografts in nude rats during perfusion in situ [30,47]. The inhibitions in hepatoma 7288CTC and ER^- MCF-7 xenografts were reversed by addition of either PTX or

8-Br-cAMP to the arterial blood containing CLA isomers, trans FAs or 9-HODE, suggesting that inhibitory GPCRs are responsible. These responses were qualitatively identical to those observed following treatment with either n-3 FAs or melatonin.

The potencies for inhibition of FA uptake in hepatoma 7288CTC differed, as follows: among the CLA isomers, t10,c12-CLA>9-HODE>t9,t11-CLA; for the trans FAs, linoelaidic>vaccenic>elaidic acids. A 50% inhibition of FA uptake and [^{3}H]thymidine incorporation in hepatoma 7288CTC and in ER$^-$ MCF-7 xenografts occurred at an arterial blood plasma concentration of about 20 μM t10,c12-CLA [25,30]. Other CLA isomers (c9,t11-, 13-HODE, c9,c11- and c11,t12-CLA) had either a lesser or no effect on FA uptake. Similar potencies for the CLA isomers were observed in ER$^-$ MCF-7 human breast cancer xenografts [30,47].

5. Proposed mechanisms for LA-dependent tumor growth and for the inhibitions caused by n-3 FAs, melatonin, CLA isomers, trans FAs and 9-HODE

5.1. Mechanism for LA-dependent tumor growth

The signaling pathway proposed to operate during LA-dependent growth in cells of solid rodent tumors and human cancer xenografts in vivo is depicted in Fig. 2A. During active tumor growth, the inhibitors described above are absent from the arterial blood; the binding sites of inhibitory GPCRs for these agents in the tumor plasma membrane are unoccupied. Intratumor cAMP concentrations are high, and FA transport, which requires cAMP, is rapid. Mechanisms that lead to the elevation of intratumor cAMP are not known but may involve activation of adenylyl cyclase by stimulatory GPCRs (as depicted in Fig. 2A) and/or by inhibition of phosphodiesterase activity. Fatty acid transport protein 1 (FATP1) is overexpressed in hepatoma 7288CTC [24] and is proposed to be the major FA transporter in these tumors. It is closely associated with long-chain acyl-CoA synthetase (LCACS), which appears to play a role in vectorial FA transport/acylation [65,66]. However, there is as yet no direct evidence that either FATP1 or LCACS contributes to FFA transport in these tumors.

Different activities of protein-mediated FA transport and/or LCACS may explain the efficiencies for FA uptake (as % of the supply rate) among the tumors shown in Fig. 1: 40–50% in hepatoma 7288CTC [25]; 25–30% in ER$^-$ MCF-7 [27], PC3, FaDu and CFDT1 xenografts; and 8% in ER$^+$ MCF-7 xenografts [29]. Possibly, the efficiency of tumor FA uptake determines tumor growth rates. The mitogen, 13-HODE, is generated by 15-lipoxygenase-1 [64] from LA removed from the arterial blood [21]. It is rate-limiting for [^{3}H]thymidine incorporation [25,29] and increases tumor growth by attenuating dephosphorylation of epidermal growth factor receptor [67]. 13-HODE also stabilizes the phosphorylated forms of mitogen-activated protein kinases (MAPK) [25,29,48]. These changes act to promote tumor growth.

5.2. Mechanism for control of LA-dependent growth by n-3 FAs, melatonin, CLA isomers and trans FAs

n-3 FAs, melatonin, active CLA isomers or active trans FAs in the arterial blood (Fig. 2B) bind to and activate their respective inhibitory GPCRs in the tumor cell membrane. The α_i subunit is released, inhibits adenylyl cyclase activity and causes an abrupt, dose-dependent suppression of intratumor cAMP. The stimulative effect of cAMP (or PKA) on FATP1/LCACS activity is removed, and LA uptake and metabolism are reduced. Formation of 13-HODE becomes rate-limiting for phosphorylation of MEK1/2 and ERK1/2, and incorporation of [^{3}H]thymidine into tumor DNA is decreased, effectively blocking the LA-dependent tumor growth stimulation (Fig. 2A). The effect of the inhibitors may be reversed by (i) removal of the agent from the arterial blood; (ii) addition of a specific antagonist (S20828, for the inhibition induced by melatonin) of the inhibitory GPCR; or (iii) by restoration of the intratumor cAMP content (by PTX or 8-Br-cAMP). Addition of 13-HODE restored downstream events, MAPK activation and [^{3}H]thymidine incorporation, but had no effect on FA uptake.

6. Topics for future research

6.1. Molecular mechanisms for FA uptake in solid tumors

Significant gaps exist in the understanding of the signaling pathways depicted in Fig. 2A and B. The physiological processes for FA uptake and for its control in solid tumors are complex and remain to be elucidated. Biochemical evidence, collected largely from in vitro experiments, indicates that fatty acids may pass through lipid vesicles and cell plasma membranes via simple diffusion [68,69]. Proponents of this mechanism suggested that the movement of FFAs across the leaflets of the plasma membrane is sufficiently fast to support the observed rates of fatty acid uptake in cells [69]. Other experiments indicated that diffusion of FFA through membrane leaflets is a more complex process and could become rate-limiting for FFA transport in cells [70]. Evidence suggested that FFA transport is highly regulated, ATP dependent and requires specific membrane transport proteins [71]. Three different membrane FFA transport proteins were identified, and evidence was presented that each may be involved in FFA transport ([65], review). Protein-mediated FFA transport may be coupled with acylation catalyzed by LCACS [65,66,72]. This coupling could provide the driving-force that moves FFAs into the intracellular space, aids in channeling FAs to specific intracellular locations and controls the rate of protein-mediated FA transport [65,66,72]. At the present time,

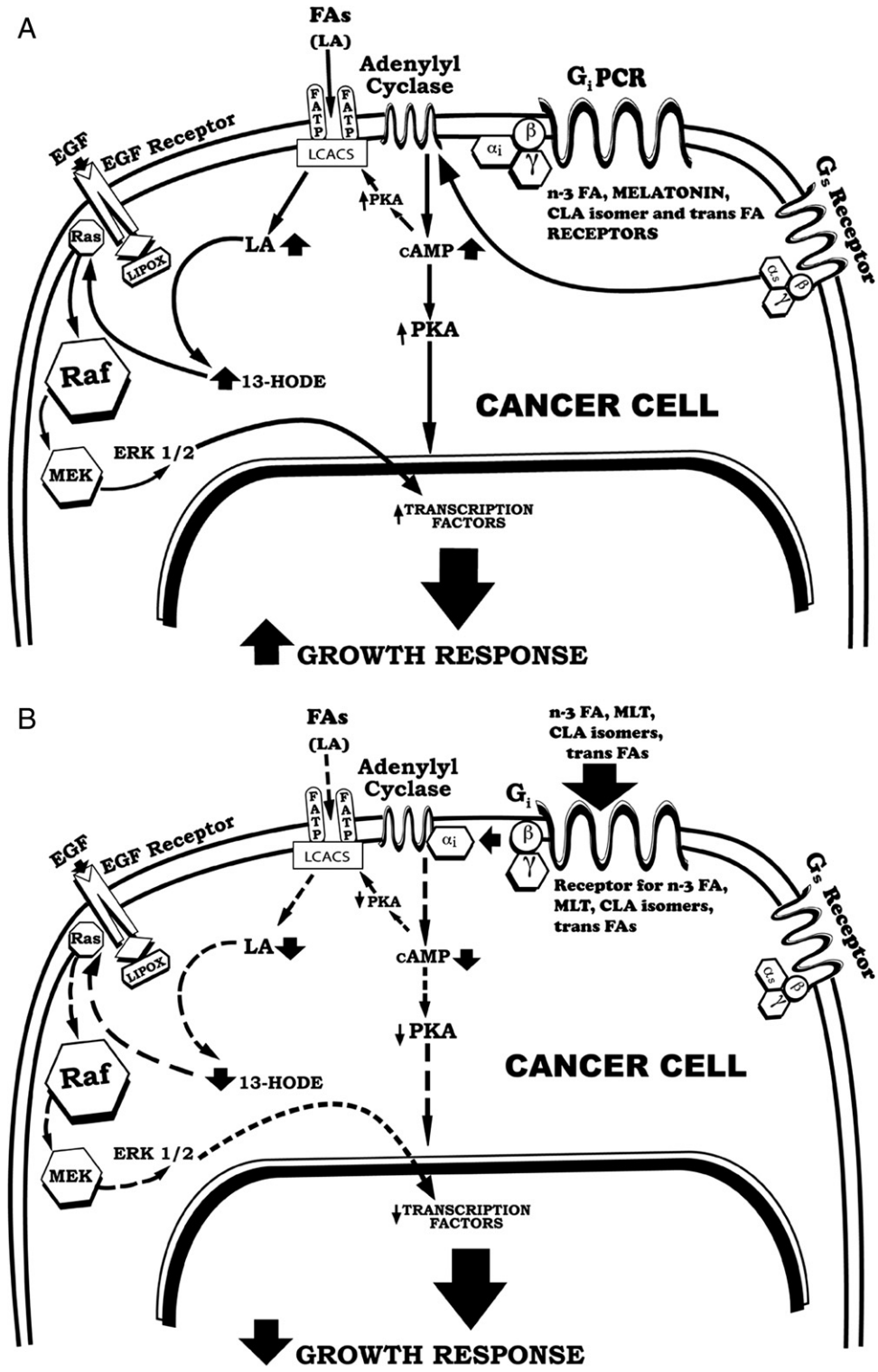

Fig. 2. Depictions of the provisional signaling pathways during LA-dependent tumor growth and during inhibition of LA-dependent growth by n-3 FAs, melatonin, CLA isomers or trans FAs. (A) The sequence of events associated with growth stimulation that follows enhanced uptake of LA and 13-HODE production is designated by solid arrows. (B) Growth suppression caused by binding of n-3 FAs, melatonin, CLA isomers or trans FAs to their respective G_iPCRs, deactivation of adenylyl cyclase, FA uptake and 13-HODE formation and other attenuated steps are designated by dashed arrows. See text for discussion.

these controversial results do not yield insights into a biochemical mechanism for control of FA uptake by intratumor cAMP in solid tumors. Considerations for such a control mechanism, however, appear to be more compatible with protein-mediated transport (Fig. 2A) rather than simple diffusion.

6.2. Role of GPCRs and intratumor cAMP in tumor growth inhibition

Of the identified inhibitors of FFA transport, only the effects of melatonin are known to be mediated by the inhibitory melatonin GPCRs, MT_1 and MT_2. mRNAs for these GPCRs are expressed in rat hepatoma 7288CTC and ER^+ MCF-7 and ER^- MCF-7 human breast cancer xenografts [27,56]. The inhibition by melatonin of intratumor cAMP content [27] and FA uptake [24,27] is reversed by the specific melatonin receptor antagonist, S20928 [24], strong evidence that these effects are mediated via the melatonin GPCRs. Inhibition of FA uptake and reduction of intratumor cAMP by n-3 FAs, t10,c12-CLA, 9-HODE, elaidic and vaccenic acids, rosiglitazone and eicosatetraynoic acid in solid tumors are not reversed by S20928, indicating that GPCRs other than the melatonin receptors are involved. Tisdale and Beck [73] were the first to describe a reduction of intracellular cAMP following addition of EPA to murine epididymal adipocytes in vitro. This effect of EPA is reversed by PTX, suggesting that the actions of EPA are mediated by an unidentified inhibitory GPCR [74].

GPR40 is an attractive candidate for a GPCR in tumor cells because many of the agents capable of affecting tumor growth described above were reported to be ligands for this receptor in cell lines in vitro [75,76]. mRNA for GPR40 was expressed in MCF-7 breast cancer cells in vitro; both LA and oleic acid activated the release of intracellular Ca^{2+} in MCF-7 cells, and this effect was partially PTX sensitive [77]. In MDA-MB-231 breast cancer cells transiently transfected with a plasmid expressing human GPR40, addition of oleic acid to the culture medium increased cytosolic Ca^{2+} within 10 s, stimulated Akt phosphorylation in 5 min and increased [^{3}H]thymidine incorporation; the effect of oleic acid on [^{3}H]thymidine incorporation was inhibited by PTX [78]. Prior to the addition of oleic acid, the MDA-MB-231 cells were subjected to a 24-h starvation period in medium without serum [78]. Previously, these authors had demonstrated that MDA-MB-231 breast cancer cells (not transfected with a plasmid expressing human GPR40) showed an increased [^{3}H]thymidine incorporation following addition of LA and oleic acid and DHA and arachidonic acid to the incubation medium [79]. 9-HODE, a potent inhibitor of intratumor cAMP content and FA uptake in hepatoma 7288CTC in vivo, has also been reported to be a ligand for GPR40 [75]. Thus, it is not clear whether GPR40 could be responsible for both tumor growth activation by LA and oleic acid and tumor growth inhibition by 9-HODE. However, 9-HODE was reported to be a ligand for the GPCR G2A, which is expressed in lymphoid tissues and macrophages [80]. In CHO-K1 or HEK293 cells stably expressing G2A, 9-HODE increased intracellular Ca^{2+} and inhibited cAMP accumulation and MAPK activation. These effects of 9-HODE were partially reversed by PTX. Most interesting, LA, arachidonic acid and 13-HODE were not ligands for receptor G2A in CHO cells [80].

The GPCR GPR120, which is abundantly expressed in lung and in mouse and human intestinal tract, was shown to be a receptor for n-3 PUFAs, in particular α-linolenic and DHA [81]. In HEK293 cells transiently expressing mouse GPR120, α-linolenic acid had no effect on cAMP production and increased the amount of phosphorylated ERK [81]. These results are opposite from the effects of n-3 FAs observed in solid rat hepatoma 7288CTC [49] and ER^+ MCF-7 human breast cancer xenografts in vivo [29]. However, when taken together these studies provide strong evidence that GPCRs will likely play important roles in signaling pathways for cancer growth and prevention ([82], review). Clearly, further research is needed to identify and characterize the GPCRs in rodent and human cancers.

7. Cell signaling pathways developed from studies in vitro

A large number of investigations have been conducted to examine the effects of n-6 and n-3 FAs, melatonin and CLA isomers on growth of normal and tumor cells in vitro. Some studies that have identified specific changes in signaling molecules are summarized below. Often, cell lines under study in vitro are incubated for a period of time in the absence of serum prior to the addition of the test agent (see Section 6.2. above). The purpose of this procedure is to down-regulate cell functions and/or to deplete endogenous lipid stores. A supply of plasma FAs and other dietary factors is continuously available to a solid tumor in fed animals; therefore, the results developed from in vitro and in vivo experiments may not be directly comparable.

7.1. Growth stimulation by n-6 FAs in vitro

In porcine vascular endothelial cells, addition of LA to the culture medium activated Akt and ERK1/2 expression after 3 to 6 h of incubation; p38 MAPK was activated after 10 min of exposure to LA, suggesting that activation of this protein kinase occurred upstream of the ERK1/2 pathway [83]. Linoleic acid also caused dose-dependent stimulation of expression of vascular cell adhesion molecule-1 mRNA (within 1 h) and protein in human microvascular endothelial cells [84]. Expression of cyclin D1 mRNA was increased within 2 h and became maximal in 5 h in T47D human breast cancer cells after the addition of arachidonic acid to the culture medium. This rise in cyclin D1 mRNA was associated with an increase in the proportion of cells in the S phase and with a stimulation of [^{3}H]thymidine incorporation [85].

7.2. Growth inhibition by n-3 FAs, melatonin and CLA isomers in vitro

7.2.1. n-3 Fatty acids

Docosahexaenoic acid inhibited growth and increased apoptosis in SK-Mel-110 metastatic human melanoma cells

in culture, changes that were associated with a hypophosphorylation of pRb and cell-cycle arrest [86]. In murine KLN-205 squamous cell carcinoma cells, addition of EPA to the medium caused a rapid release of intracellular Ca^{2+} stores; inhibited translation initiation, protein and DNA synthesis; and arrested cell cycle progression and cell proliferation [87]. Eicosapentaenoic acid also decreased cyclin D1 expression and blocked cell-cycle progression and growth in solid KLN-205 tumors in mice [87]. Addition of EPA and DHA to the culture medium inhibited growth of MDA-MB-231 human breast cancer cells relative to the control oleic acid, which had no effect [88]. In MDA-MB-231 cells synchronized by serum starvation, both n-3 FAs caused a concentration-dependent delay in movement from G2/M to G0/G1, relative to the control oleic acid. This delay was correlated with a decreased activation of CDK1-cyclin B1 [88]. In MCF-7 human breast cancer cells, addition of n-3-enriched LDL was reported to up-regulate expression of the proteoglycan, syndecan-1 [89]. When added as the free acid bound to bovine serum albumin, EPA had no effect, but albumin-bound DHA was as effective as n-3-enriched LDL [89]. In Caco-2 human colon cancer cells, DHA was reported to down-regulate expression of inducible nitric oxide synthetase, cGMP, and to up-regulate cyclin-dependent kinase inhibitors [90]. Both EPA and DHA were reported to reduce expression of vascular endothelial growth factor and cyclooxygenase-2 and to inhibit ERK1/2 phosphorylation in HT-29 human colon cancer cells [91]. In a hippocampal slice preparation obtained from male mice, n-3 FAs suppressed several protein kinase activities, including PKA, PKC, Ca^{2+}/calmodulin-dependent kinase and MAPK [92].

7.2.2. Melatonin

The actions of melatonin on cell proliferation in rodent and human cancer cell lines in vitro were recently reviewed [56,93]. The reader is referred to these reviews for further details. Most in vitro studies have indicated that addition of physiological plasma melatonin levels (0.1 to 1 nM) to rodent or human cancer cells suppressed cell proliferation. Higher concentrations may be cytostatic or cytotoxic. A central mechanism by which melatonin may influence cell signaling events for tumor cell proliferation in vitro is via inhibition of cAMP accumulation initiated by activation of the inhibitory melatonin GPCRs, MT_1 and MT_2. These receptor subtypes have been cloned and identified in many rodent and human tumor cell lines. One, both or none of the melatonin GPCR subtypes may be present in a tumor cell line. Neither MT_1 nor MT_2 was detected in ER^- MDA-MB-231 human breast cancer cells, which may explain why physiological melatonin levels failed to inhibit cell proliferation in these cells. In MCF-7 cells, melatonin decreased tumor cell proliferation by delaying progression from G1 to the S phase of the cell cycle in vitro. PTX and melatonin GPCR antagonists blocked the actions of physiological levels of melatonin on intracellular cAMP levels and growth.

Melatonin is also an important antioxidant [56] and these antioxidant properties may also influence cell proliferation. In rat C6 glioma cells, addition of 1 mM melatonin to the incubation medium was reported to inhibit cell progression from G1 to S phase, cell proliferation and phosphorylation of Akt but not ERK1/2 [94]. Lower melatonin levels (1 nM to 10 μM) had no significant effect on cell proliferation in C6 glioma cells. The inhibition of cell proliferation by 1 mM melatonin was not reversed by either a melatonin receptor antagonist or PTX, suggesting that the actions were melatonin receptor independent [94].

7.2.3. CLA isomers and trans FAs

Studies performed in vitro reported the effects of purified c9,t11-CLA in human HT-29 and Caco-2 colon cancer cells [95]. The CLA isomer inhibited cell proliferation with an IC50 of 35 μM in HT-29 cells and 109 μM in Caco-2 cells. The trans FA, vaccenic acid, had no effect on cell proliferation. In HT-29 cells, c9,11t-CLA also suppressed expression of mRNA for c-myc, c-jun, cyclin D1 and peroxisome proliferator-activated receptor δ. [95]. The effects of c9,t11- and t10,c12-CLA isomers were tested in human PC-3 prostate cancer cells [96]. Both isomers inhibited cell proliferation, but t10,c12-CLA was more effective. Expression of bcl-2 gene was decreased and $p21^{WAF/Cip1}$ mRNA levels were increased by t10,c12-CLA; c9,t11-CLA had no effect. Rather, c9,t11-CLA decreased 5-lipoxygenase expression. It was concluded that t10,c12-CLA affected cell-cycle control and that c9,t11-CLA affected arachidonic acid metabolism in PC-3 cells [96]. However, in a mouse mammary tumor cell line 4526, t10,c12-CLA, but not c9,t11-CLA, reduced cell proliferation and cell viability and induced apoptosis by decreasing formation of the 5-lipoxygenase product, 5-hydroxyeicosatetraenoic acid [97]. Cell viability in cells treated with t10,c12-CLA was returned by adding back 5-hydroxyeicosatetraenoic acid [97]. In MCF-7 human breast cancer cells, it was observed that CLA isomers reduced cyclooxygenase-2 (COX-2) expression induced by 12-*O*-tetradecanoylphorbol-13-acetate, a proinflammatory agent [98]. Binding studies indicated that t10,c12-CLA was more effective than c9,11t-CLA in reducing binding of c-Jun to the COX-2 cAMP response element. Overexpression of c-Jun reversed the inhibitory effect of both CLA isomers on COX-2 transcription [98].

8. Conclusions and prospects

Substantial progress has been made toward defining the mechanisms by which n-6 FAs stimulate and the dietary factors, n-3 FAs, melatonin, CLA isomers and trans FAs, suppress growth in rodent tumors and human cancer xenografts in vivo and in vitro. A sequence of signaling molecules participate, from cell surface FA receptors and transporters, adenylyl cyclase, cAMP, intracellular Ca^{2+} release, lipid mediators, protein kinases, transcription

factors and synthesis of new mRNAs leading to synthesis of new proteins. Mechanisms developed from experiments in tumor cell lines in vitro have mostly highlighted signaling molecules toward the end of the sequence, whereas the experiments performed in vivo in solid tumors have revealed earlier steps, e.g., transport of n-6 FAs and its regulation by inhibitory GPCRs and cAMP. Linoleic acid and arachidonic acid uptake and generation of lipid mediators from these FAs, which may stimulate tumor growth, are controlled by FA transport. The rate of transport of n-6 PUFAs, monounsaturated and saturated FAS is controlled by inhibitory GPCRs, which is down-regulated by n-3 FAs, melatonin, CLA isomers and trans FAs. However, uptake of n-3 FAs, CLA isomers and trans FAs does not appear to be controlled by the same transporters or by GPCRs or cAMP. The transporters for n-6 PUFAs, monounsaturated and saturated FAs and the inhibitory GPCRs that regulate this transport and control growth in rodent or human cancer xenografts remain to be determined. Mechanisms for transport of n-3 FAs, CLA isomers and trans FAs are not known either. A goal of this review is to increase interest in and study of this signaling process. Further research will certainly lead to the identification and characterization of these proteins and to a clearer definition of their role in the initial and downstream events in this signaling sequence in tumors.

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