Leukotriene B4 Creates a Favorable Microenvironment for Murine Melanoma Growth

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Abstract
Chronic inflammation has long been associated with neoplastic progression. Our group had recently shown that the addition of a large number of apoptotic tumor cells to the tumor microenvironment induces a potent acute inflammatory reaction capable of promoting melanoma growth; however, primarily necrotizing cells do not cause such a reaction. Here, we show that potent inflammatory agents, such as lipopolysaccharide (LPS) and carrageenan, also promote growth of subtumorigenic doses of melanoma cells, having no effect on melanoma proliferation in vitro. Inhibition of 5-lipoxygenase (5-LOX) seems to have a pivotal role in this model because caffeic acid and MK886, a FLAP (5-LOX–activating protein) inhibitor, partially hindered tumor growth induced by apoptotic cells or LPS. Other enzymes of the arachidonic acid pathway, cyclooxygenase-1 and cyclooxygenase-2, seem to have no participation in this tumor promoter effect, as the inhibitor of both enzymes (indomethacin) did not alter melanoma growth. Leukotriene B4 (LTB4), the main product of the 5-LOX pathway, was able to induce growth of subtumorigenic inocula of melanoma cells, and a LTB4 receptor antagonist inhibited acute inflammation-associated tumor growth. Addition to the tumor inflammatory microenvironment of eicosapentaenoic acid, an ω3-polyunsaturated fatty acid with anti-inflammatory properties, or leukotriene B5, an eicosapentaenoic acid–derived leukotriene, significantly inhibited tumor development. These results give new insights to the mechanisms through which inflammation may contribute to tumor progression and suggest that LOX has an important role in tumor progression associated with an inflammatory state in the presence of apoptosis, which may be a consideration for apoptosis-inducing treatments, such as chemotherapy and radiotherapy. (Mol Cancer Res 2009;7(9):1417–24)

Introduction
The association between inflammation and cancer was noted by Rudolph Virchow in 1858, when he described for the first time the presence of a leukocytic infiltrate in tumor tissues and concluded that cancers frequently occurred at sites of chronic inflammation (1). The contribution of environmental factors to cancer progression is becoming the focus of intense research, and special attention has been devoted to chronic inflammatory diseases that are associated with a high prevalence of tumors (2, 3). Those include common conditions such as gastritis, esophagitis, prostatitis, and chronic skin ulcers, among others. Although several of these pathologies are associated with viral or bacterial infections, many others are not, and the mechanisms through which cancer develops in the inflammatory microenvironment are only beginning to be uncovered.

Opposing effects of inflammation on cancer have been described. Acute inflammation is believed to induce an antitumor immune response, whereas chronic inflammation seems to facilitate neoplastic growth (4). Very few clinical reports corroborate the first assumption, which seems to have been mostly based on animal studies of tumor immunity, whereas several epidemiologic, clinical, and experimental studies in humans as well as experimental studies in animals confirm the second postulate (5).

Eicosanoids, 20-carbon lipid-derived effectors of the inflammatory response, play a central role in carcinogenesis promotion and environmental support of neoplastic progression (6). These molecules, comprising several different classes and functions, are mostly derived from membrane-associated lipids, particularly arachidonic acid (AA), which is an ω6 polyunsaturated fatty acid (PUFA). There are two major metabolic pathways of interest in the AA cascade: the cyclooxygenase (COX) and the lipoxygenase (LOX) pathways. Both pathways have direct effects on carcinogenesis induction and apoptosis suppression (7, 8). Furthermore, both inflammatory pathways are linked indirectly to the induction and promotion of tumor growth through their actions on the microenvironment, such as induction of angiogenesis and growth factor production (9).

Apoptosis, or programmed cell death, occurs at high rates in infected, inflamed, or remodeling tissues. Free apoptotic cells are rarely found in tissues, even during inflammation, as they...
are rapidly removed by either local nonprofessional phagocytes or specialized phagocytes such as macrophages (10). Recognition, uptake, and degradation of apoptotic cells by phagocytes occur extremely fast, preventing inappropriate exposure of adjacent tissues to the intracellular contents of the cells undergoing apoptosis. A hallmark of the removal of apoptotic cells in vivo is the failure to generate an associated inflammatory response. Thus, depending on the context, the removal of apoptotic cells may induce an inhibitory effect that can antagonize a coincident proinflammatory response. In contrast, the uptake of necrotic cells results in activation of the inflammatory response and may promote an immune response to self-antigens (11, 12).

Based on the evidence found in our laboratory (13) that showed the positive correlation between acute inflammation, induced by massive apoptosis, and melanoma progression, we extended our investigation on the inflammatory pathways involved in that model.

Results
Different Acute Inflammatory Stimuli Promote Melanoma Tumor Progression

An essential aspect in the homeostasis maintenance of a multicellular organism is the disposal of dead cells, especially apoptotic cells. Normally, this clearance process is not characterized by the induction of an inflammatory response. However, several studies have shown that the death of a great amount of cells by apoptosis in certain cases can lead to a secondary necrosis followed by the activation of an inflammatory response (14, 15). We have previously observed (13) that the coinjection of a large amount of apoptotic cells ($10^6$ γ-irradiated Tm1 melanoma cells) is associated with a local inflammatory response and promotes the growth of a subtumorigenic dose of viable melanoma cells ($10^5$ Tm1 cells; Fig. 1A). To confirm the key role of the acute inflammatory process in the growth of this subtumorigenic amount of melanoma cells, $10^5$ Tm1 cells were coinjected with known inflammatory stimuli, lipopolysaccharide (LPS) or carrageenan. Both LPS (Fig. 1B) and carrageenan (Fig. 1C) are capable of promoting the growth of this suboptimal number of tumor cells, similarly to the coinjection protocol using apoptotic and viable tumor cells (Fig. 1A). These data hint at the presence of common inflammatory pathways involved in melanoma progression induced by these inflammatory stimuli. We chose to focus on the role of apoptotic cells as an inflammatory stimulus because of potential clinical implications related to cancer treatments because radiotherapy and chemotherapy treatments induce massive apoptosis.

Apoptosis Occurs for at Least 72 Hours at the Injection Site and Is Accompanied by an Inflammatory Infiltrate

Subcutaneous tissues coinjected with apoptotic ($10^5$) and viable tumor cells ($10^3$) were surgical removed 6 hours, 24 hours, 72 hours, and 14 days after coinjection. Histologic analysis showed the presence of an intense cellular infiltrate surrounding the injection site after 6 and 24 hours, which was reduced after 72 hours and almost absent 14 days after the coinjection (Fig. 2, HE, top). A large number of dying cells, in varying stages of the process, and including necrotic debris, were also clearly noted. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analysis showed the presence of apoptotic cells diffusely located in the injection site 6 and 24 hours after coinjection. After 72 hours, the apoptotic cells are concentrated in the central region of the injection site, surrounded by viable melanoma cells. After 14 days, we observed few apoptotic areas in the tumor mass (Fig. 2, TUNEL, bottom) and very few inflammatory cells surrounding or permeating the tumor mass (Fig. 2, HE, top).

Inhibition of the 5-LOX Pathway, but not COX, Hinders Tumor Progression Induced by Acute Inflammation

The recruitment of leukocytes and production of inflammatory mediators are important elements in any inflammatory
response. The metabolic pathway of activation and production of these mediators includes the utilization of AA (an ω6 fatty acid derived from linoleic acid obtained from the diet) or eicosapentaenoic acid (EPA, an ω3 fatty acid derived from α-linolenic acid obtained from the diet), and enzymes such as LOX or COX that convert these fatty acids into leukotrienes or prostaglandins, respectively.

Animal groups submitted either to coinjection (10^6 apoptotic cells and 10^3 viable tumor cells) or injection of LPS and 10^3 viable tumor cells or injection of a tumorigenic dose of viable tumor cells only (in the concentration of 2 × 10^5 melanoma cells) were treated with indomethacin, a nonselective inhibitor of COX-1 and COX-2, caffeic acid (5-LOX inhibitor), or MK886 [FLAP (5-LOX–activating protein) inhibitor]. Figure 3 shows that the use of indomethacin does not interfere with tumor progression, as observed with the COX-2–selective inhibitor meloxicam (data not shown), suggesting that the activation of the cyclooxygenase pathway does not play an important role in growth of a subtumorigenic dose of melanoma cells induced by a large number of apoptotic cells (Fig. 3A) or by the potent inflammatory agent LPS (Fig. 3G). The anti-inflammatory activity of these inhibitors was previously confirmed by footpad inflammation assays (data not shown). However, 5-LOX pathway inhibitors, such as caffeic acid (Fig. 3B and H) or MK886 (Fig. 3C), inhibit tumor progression in this model. Dexamethasone, a glucocorticosteroid with very complex effects on inflammatory pathways (16, 17), also does not have any effect on melanoma growth in vivo or in vitro (data not shown). Tumor growth unrelated to acute inflammation is not influenced by any of these inhibitors (Fig. 3D-F).

Neutrophil recruitment to inflamed sites is stimulated by leukotriene B4 (LTB4), one of the main products of the AA/5-LOX inflammatory pathway. Inhibition of this pathway by caffeic acid, but not by COX inhibitors (data not shown), altered neutrophil recruitment kinetic to the injection site (Fig. 4A and B), confirming that, in this model, tumor growth depends on the inflammatory response elicited by the 5-LOX pathway but not on COX-mediated inflammation.

**LTB4, a Product of the 5-LOX Pathway, Is Essential for Melanoma Progression Associated with Acute Inflammation**

Leukotrienes, synthesized from AA or EPA through the 5-LOX pathway, play a major role in inflammatory reactions. Among the biologically active metabolites of this pathway, LTB4, derived from leukotriene A4 (LTA4) in the AA/5-LOX metabolic pathway, is a potent chemoattractant involved in the recruitment of neutrophils and leukocytes to the inflamed sites (18). To evaluate the role of LTB4 in this model, animals received a subtumorigenic dose of Tm1 cells (10^5 melanoma cells) admixed with 100 nmol/L of LTB4 in the subcutaneous tissue (Fig. 5A), whereas no effects of LTB4 on in vitro tumor cell proliferation were observed (Fig. 5B). Conversely, mice subjected to coinjection were treated with LTB4-ethanolamide (Eth), a LTB4 receptor 1 (BLTR1) antagonist (19). Treatment with LTB4-ethanolamide (Eth) significantly reduced tumor progression associated with inflammation (Fig. 5C), but did not present any effect on tumor growth in animals injected only with tumor cells (Fig. 5D).

**EPA, an ω3 PUFA, and Its Derived Mediator Leukotriene B5 Inhibit Melanoma Growth Associated with Inflammation**

EPA is an ω3 PUFA obtained from the diet and, similar to AA, is a substrate for COX and LOX enzymes, among others. Mediators derived from EPA present mostly anti-inflammatory properties (20) and have beneficial effects on patients with cancer (reviewed in ref. 21). To study their role in our model, groups of animals received s.c. EPA or leukotriene B5 (LTB5) close to the coinjection site. As shown in Fig. 6A and C, both significantly reduced melanoma growth induced by inflammation while presenting no effects on control groups (Fig. 6B and D).

**Discussion**

Chronic infection and inflammation have long been associated with cancer, but only in recent years has this complex correlation begun to be unraveled (22). Several experiments...
using animal models have clearly established a role for cells of the immune system in tumor progression (23) and such phenomenon in humans may be associated with a poor prognosis in the presence of tumor-associated macrophages (24), or a favorable prognosis when associated to neutrophil infiltration (reviewed in ref. 25), although several other reports do not support the last conclusion (26-31). Although human melanoma is not generally associated with overt inflammation, several recent studies highlight the importance of an inflammatory microenvironment, especially the role of the innate immunity, for melanoma initiation and progression (reviewed in ref. 32). Proteomic analysis of B16F10 mouse melanoma suggested that inflammatory proteins are overexpressed in the first 3 to 7 days of tumor challenge in C57Bl-6 mice (33), and, perhaps more interestingly, an inflammatory gene signature was able to identify with high accuracy human metastatic melanoma present in sentinel lymph nodes but not in nonmetastatic nodes (34).

Our results show that the generation of an acute inflammatory process induced by apoptotic cells, LPS, or carrageenan provides a favorable environment for the development of tumors when a subtumorigenic dose of melanoma cells is s.c. injected in mice (Fig. 1). Although apoptosis has been associated with the inhibition of inflammatory responses, several factors, including unscheduled and massive cell loss, contribute to the “decision” by macrophages of which type of response is adequate at the moment (35). Furthermore, it is believed that the type of cell death (necrotic versus apoptotic) is also determined by the type and amount of tissue injury, and concurrent apoptosis of a large number of cells, with an associated clearance deficiency, may lead to secondary necrosis and a proinflammatory response (36, 37). Effective cancer therapy (chemotherapy, radiotherapy, immunotherapy) induces activation of apoptosis programs (38), but little is known about the host response to apoptotic cells in the tumor microenvironment. Figure 2 and a previous work by our

![Figure 3](image-url)

**FIGURE 3.** Inhibition of the 5-LOX pathway, but not COX, reduces tumor progression associated with an inflammatory state. Induction of melanoma growth (10³ viable Tm1 cells) by apoptotic cells (10⁶ γ-irradiated Tm1; A) or LPS (2.5 μg/animal; G) in the coinjection arm is not abrogated by indomethacin, a COX-1/COX-2 inhibitor (20 mg/animal/d), but is reduced by treatment with caffeic acid, a 5-LOX inhibitor (25 mg/animal/d; B and H, respectively), and MK886, a FLAP inhibitor (1 mg/kg/d; C). Treatment of animals with indomethacin (D), caffeic acid (E), or MK886 (F) does not affect the tumor progression obtained with injection of 2 × 10⁵ Tm1 melanoma cells (tumorigenic dose).
group (13) show that irradiated melanoma cells undergo massive apoptosis when injected s.c., which is accompanied by a leukocytic infiltrate indicative of an inflammatory response. The inflammatory infiltrate peaks 24 hours after injection, but inflammatory cells are not present in large numbers at 14 days, when the tumor is already established (Fig. 2, top). Apoptotic cells can be found as early as 1 hour (not shown) and throughout tumor progression (Fig. 2, bottom), suggesting that the early phases of the inflammatory process are vital to the host response to the tumor and may define the tumor outcome.

Among the mediators of the inflammatory response, the metabolites of AA, such as prostaglandins, thromboxanes, and leukotrienes, have an essential role. In humans, AA is mainly metabolized by lipoxigenases [including 5-LOX, 12-LOX, and 15-LOX] and cyclooxygenases [including COX-1 and COX-2; ref. 39]. Leukotrienes are molecules derived from the catalytic action of the 5-LOX enzyme, which generates 5-HpETE and LTA4. The latter may be further metabolized through the action of LTA4 hydrolase or leukotriene C4 synthase enzymes, resulting in the production of LTD4 or leukotriene C4, respectively. 5-LOX is expressed in a limited number of cells, mainly leukocytes such as neutrophils, macrophages, mastocytes, and also by epithelial cells. LTD4 is the main inflammatory mediator originating from the 5-LOX pathway in neutrophils. This molecule is a potent chemoattractant of neutrophils and macrophages to the inflamed site and can induce angiogenesis (40). Several studies have shown that 5-LOX or its products, expressed by diverse tumor cell lineages, including melanoma, may stimulate proliferation and inhibit apoptosis (8, 41). The role of COX in carcinogenesis has been extensively studied (42), but little is known about the contribution of 5-LOX to inflammation in our model as well (Fig. 6A and C).

In our model, both COX or 5-LOX inhibitors showed no influence on Tm1 melanoma proliferation in vitro (data not shown). However, experiments in vivo showed that the inhibition of 5-LOX pathway (Fig. 3B and C), unlike the inhibition of the COX pathway (Fig. 3A), was able to reduce melanoma growth and progression associated with acute inflammation. This effect was only observed in the coinjection group, with no effect on those animals injected with a tumorigenic dose of melanoma cells (2 × 10⁵ Tm1 cells). Nevertheless, the scientific literature at large does not consider the injection of tumorigenic cells an inflammatory stimulus, in accordance with our own previous

**FIGURE 4.** Neutrophil infiltrate persists after 5-LOX pathway inhibition. The inhibition of 5-LOX (caffeic acid; 25 mg/animal/day) does not inhibit the neutrophil infiltrate in mice submitted to coinjection of apoptotic cells (10⁶ γ-irradiated Tm1) or a subtumorigenic dose of Tm1 (10⁵ viable Tm1 cells; A, black circles and B, bottom). Subcutaneous tissues from nontreated (PBS) and treated (caffeic acid) animals were surgically removed 6, 24, and 72 h after coinjection and processed for immunohistochemistry analysis using a specific antibody against myeloperoxidase, an antigen expressed mainly by neutrophils (×100). *, P < 0.05, statistical significance using nonpaired nonparametric Student’s t tests.
work (13) that showed no inflammatory infiltrate in the tissue surrounding the injection site of a large amount of viable melanoma cells.

Melanoma is a notoriously hard disease to manage in later stages because of an elevated resistance to either chemotherapy or radiotherapy. Our results show that lipid mediators derived from the AA/5-LOX metabolism have a fundamental stimulatory role in melanoma progression associated with inflammation, whereas ω3 PUFA-derived metabolites have opposite effects, and that the early host response to cellular death may be a determinant of the final outcome of this deadly disease. Because the same mechanisms that promote progression of a primary tumor contribute to the establishment of metastatic disease, our findings can be extrapolated to the later phases of melanoma. Because the current approaches for cancer treatment (chemotherapy, radiotherapy, and immunotherapy) induce massive apoptosis in the target tissue, these results may have implications in the understanding of cancer treatment failures. The apoptosis caused by cancer treatment might induce an inflammatory reaction that can promote the proliferation of few surviving cells at the treated site. Rapid tumor relapses observed after tumor treatment might be related to these mechanisms, which warrant further investigation.

Materials and Methods

Cell Culture, Reagents, and Proliferation Assay

The murine melanoma cell line Tm1 (52) was cultured in RPMI (pH 6.9; Gibco) supplemented with 5% FCS (Gibco) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cell proliferation was determined using a standard MTT assay (Sigma) following standard protocol (53). LPS and carrageenan were purchased from Sigma; caffeic acid and MK886 were purchased from Calbiochem; indomethacin was purchased from Merck Sharp and Dohme; LTB4 and LTB4-ethanolamide were purchased from Cayman Chemical; EPA was purchased from Calbiochem; and leukotriene B5 was purchased from Sigma.

Tumorigenicity Assays

Subconfluent monolayers of Tm1 melanoma cells were harvested after trypsin treatment, counted, and resuspended in culture media at a concentration of 2 × 10^5 cells/mL. Another suspension of 2.10^7 cells/mL was submitted to γ-irradiation (200 Gy) for induction of apoptosis, using GammaCell 300 Elan (Nordion Internacional, Inc.). After the irradiation procedure, both cell suspensions were centrifuged and resuspended in PBS (140 mmol/L NaCl, 2 mmol/L NaH2PO4, 8 mmol/L Na2HPO4) and 50 μL of each solution (1 × 10^5 viable cells plus 1 × 10^6 apoptotic cells) were admixed and coinjected s.c. in the flank of syngeneic 6- to 8-wk-old C57Bl/6 female mice. To assess the effect of LPS or carrageenan in tumor progression, animals were cotreated with a mixture of 10^5 viable Tm1 cells (50 μL) and LPS (2.5 μg) or carrageenan (0.1%). Animals were kept under 12-h daylight cycles, without food restriction, and checked daily for tumor development. Each experimental group consisted of at least five animals. Tumor growth was monitored three times per week and tumor volume was determined as follows: [maximum diameter × (minimum diameter)^2]/2 (54). Mice with a subcutaneous mass >20 mm³ were considered positive for the presence of tumors.

Histologic and Immunohistochemistry Analysis

Mice were sacrificed according to institutional guidelines, and subcutaneous tissues corresponding to the site of coinjection were excised in different periods after injection (6 h, 24 h, 72 h, and 14 d). Samples were submitted to histologic analysis after paraffin embedding and H&E staining. Hematoxylin-stained sections were used for immunohistochemistry analysis.

FIGURE 5. LTB4 is an important positive mediator of melanoma progression induced by inflammation. LTB4 (100 nmol/L) is able to promote the growth of a subtumorigenic dose of Tm1 melanoma cells (10^5 Tm1 cells; A) but does not promote in vitro Tm1 proliferation (B). The LTB4 receptor 1 antagonist, LTB4-ethanolamide (Eth; 100 nmol/L/animal/d) significantly inhibits melanoma tumor growth in mice coinjected with apoptotic Tm1 melanoma cells (10^5 γ-irradiated Tm1) and a subtumorigenic dose of Tm1 (10^5 viable Tm1 cells; C), but not in mice injected only with a tumorigenic dose of Tm1 melanoma cells (2 × 10^5 cells; D). *, P < 0.05, statistical significance using nonpaired nonparametric Student's t tests.
for neutrophil detection (myeloperoxidase-specific antibody, Santa Cruz Biotechnology). TUNEL reactions were done on serial sections using an in situ cell death detection kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit S7100, Chemicon International; ref. 55) according to the manufacturer’s instructions. Sections were visualized in a Zeiss Axioskop40 microscope, and images were captured using a Sony Cybershot 3.3 megapixels and analyzed in a MetaVue Imaging System v.6.1 (Molecular Devices Corporation) for cell counts.

In vivo Treatment with Modulators of Inflammatory Pathways

Indomethacin, diluted in sterile PBS, was used at a concentration of 2 mg/kg of weight for each mouse. The treatment program consisted in 10 i.m. doses (100 μL) daily, the first one at the same time of tumor cell injection. MK886 was used at a concentration of 1 mg/kg of weight for each mouse, and the treatment program consisted in six i.p. injections (200 μL) daily, the first one at the time of tumor cell injection. Caffeic acid treatment consisted in 10 i.p. injections (2.5 mg/500 μL/animal) the first one at the moment of injection. For analysis of the effect of LTB4 in melanoma tumor progression, animals were s.c. injected with a 100 μL suspension containing 10^5 Tm1 cells admixed with LTB4 (100 nmol/L final concentration). In addition, a group of animals was treated with LTB4-ethanolamide, an antagonist of LTB4 receptor 1 (BLTR1; 100 nmol/L final concentration), at the moment of injection and for 4 more days, in the neighboring injection site through the s.c. route. EPA (50 μmol/L final concentration) and LTBS (100 nmol/L final concentration) treatment was similar to LTB4-ethanolamide. All experiments were done using groups of five to ten 6- to 8-wk-old female mice. Control groups received either injections of PBS or a subtumorigenic dose of Tm1 melanoma cells (10^3) according to the type of experiment (see Results and figure legends). All doses of anti-inflammatory agents were based on published studies (56, 57), and the anti-inflammatory activity of these compounds was confirmed in vivo using the murine footpad inflammation model.

Statistical Analysis

The data are expressed as the mean ± SD. Nonpaired parametric Student’s t test was used to analyze the difference between the means using the InStat software package. Kaplan-Meier survival analyses by the Mantel-Haenszel log-rank test was done by using GraphPad Prism version 4 for Windows (GraphPad). The significance level was established at P < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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