Inhibition of Human Malignant Neuroblastoma Cell DNA Synthesis by Lipoxygenase Metabolites of Arachidonic Acid¹

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ABSTRACT

In vivo studies have shown that inhibitors of cyclooxygenase metabolism of arachidonic acid may diminish growth and metastasis of certain tumors. Because cyclooxygenase inhibition may increase the production of lipoxygenase products of arachidonic acid metabolism, we have investigated the effect of two such products, 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-hydroxyeicosatetraenoic acid (15-HETE) on tumor cell proliferation in vitro.

When neuroblastoma cells (SK-N-SH) in culture were treated with 12-HETE for 18 hr, incorporation of [3H]thymidine was inhibited up to 64% at concentrations from 20 to 50 μM. Under the same conditions, 15-HETE resulted in inhibition of up to 46%, while arachidonic acid had no apparent effect. When evaluated in the presence of serum, 12-HETE at a concentration of 120 μM produced a 20.6 ± 2.8% (S.E.) inhibition of the increase in total DNA content over 48 hr, while 15-HETE at this concentration produced a 16.5 ± 5.3% inhibition. We conclude that 12-HETE, the product of platelet lipoxygenase, and 15-HETE, a product of neutrophil and lymphocyte lipoxygenases, can inhibit human neuroblastoma cell growth in vitro and may play a role in the effect of cyclooxygenase inhibitors on tumor growth in vivo.

INTRODUCTION

The role of products of arachidonic acid metabolism in the spread and growth of malignant cells remains unclear, despite considerable research in the past decade (23). Arachidonic acid is metabolized to prostaglandins via the enzyme cyclooxygenase or to HPETEs² via the enzyme lipoxygenase (9, 19). HPETEs are subsequently reduced to HETEs.

The cyclooxygenase derivatives of arachidonic acid, prostaglandins, thromboxane, and prostacyclin, have crucial functions in the physiology of many cell systems. Animals treated with inhibitors of cyclooxygenase, indomethacin, or aspirin (27), prior to inoculation with tumor cells, became relatively resistant to tumor growth (3, 18, 21) and metastasis (6, 14). While these results might suggest that prostaglandins promote tumorigenesis, it is possible that lipoxygenase products, which can be elevated by cyclooxygenase inhibitors (16), may have an antitumor effect. Recent findings with human platelets support this hypothesis. Stimulated normal human platelets produce 12-HETE in as great abundance as thromboxane (17). Schafer found that platelets from patients with myeloproliferative disorders, including chronic myelogenous leukemia, had a deficiency of lipoxygenase activity (26). Subsequently, Ibele et al. (13) reported that unidentified products of platelet metabolism inhibited the proliferation of malignant melanoma cells in tissue culture. The effect was found to persist following cyclooxygenase inhibition but was not present when platelets from a patient with a myeloproliferative disorder were used in lieu of control cells. These findings together suggest that 12-HETE might be a factor responsible for inhibition of proliferation of melanoma cells.

We have investigated the effects of HETEs on tumor cell DNA synthesis in vitro. Neuroblastomas are known to undergo differentiation either spontaneously or in response to chemotherapy (22). In vitro, neuroblastoma differentiation is affected by lipid mediators such as butyric acid and prostaglandin E (24) and by antioxidants such as vitamin E and ascorbic acid (25). We now report that 12-HETE, the predominant product of platelet lipoxygenase (20), and 15-HETE, a product of T-lymphocyte (8) and neutrophil (2) lipoxygenases, are inhibitory to DNA synthesis and thymidine incorporation in human neuroblastoma cells in tissue culture.

MATERIALS AND METHODS

Preparation and Purification of 12-HETE and 15-HETE. 12-HETE was prepared by incubating 2 mM arachidonic acid (Nu-check Prep, Elysian, MN) in 1 liter of Tris-HCl (pH 8.0), 10 mM EDTA, and 30 μM indomethacin with washed freshly outdated human platelets from 20 units of platelet concentrates. 15-HETE was prepared by incubating arachidonic acid with soybean lipoxygenase (Sigma Chemical Co., St. Louis, MO) essentially as described by Crawford et al. (5). Both preparations were extracted with chloroform after acidification, treated with triphenylphosphine to reduce hydroperoxides, and initially purified by triplicate chromatography with hexane:diethyl ether mixtures of 100:0, 90:10, and 80:20. The hydroxyacid-enriched fractions were pooled and then reduced with sodium borohydride to ensure the absence of hydroperoxides. The HETEs were finally purified by preparative HPLC using a Beckman C-18 reverse-phase preparative column eluted with methanol:water (70:30, v/v) at a flow rate of 3 ml/min. Purity was greater than 95% by analytical HPLC monitoring of trimethylsilyl ether, methyl ester derivatives. Both preparations were further purified on HPLC and thin-layer chromatography, and by GC-mass spectroscopy of trimethylsilyl ether, methyl ester derivatives. Both 12-HETE and 15-HETE yielded single peaks with mass spectra identical to published spectra (30). Purity was greater than 95% by analytical HPLC monitoring at 206 nm. Concentrations were determined from UV spectra using a molar extinction coefficient of A206 of 30,500 (11).

Neuroblastoma Cell Culture. SK-N-SH Neuroblastoma cells were obtained from a line established at Memorial Sloan-Kettering Cancer Center from a child with metastatic disease (1). The cells were maintained in RPMI-1640 medium with 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) added (complete medium). The cultures were incubated in plastic tissue culture flasks (Corning Glass Works, Corning, NY) in a humidified atmosphere of 95% air and 5% CO₂. Tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, NY.
Cell viability was determined by trypan blue dye exclusion (25) and by [3H]thymidine labeling of cells. After the indicated treatment (e.g., growth for 18 hr with or without serum supplementation), cells were incubated in complete medium containing [3H]thymidine (0.10 μCi/ml) for 24 hr (approximately 1.2 population doubling times). Cells were then washed, fixed with 1% paraformaldehyde:1% glutaraldehyde:100 mM phosphate buffer, pH 7.2, and overlayed with NTB2 nuclear track emulsion (Kodak, Rochester, NY). After a 4-week exposure, they were developed and scored for [3H]thymidine incorporation. 100 cells in 10 random 400× fields were scored and recorded. Cells were scored as positive for [3H]thymidine incorporation if cell-associated grains were greater than 500% of background.

**Cellular Proliferation Assays.** To quantify tumor cell proliferation, both [3H]thymidine and DNA content assays were performed. These assays were used because single-cell suspensions, which would allow accurate cell counts, could not be obtained. Twenty-four hr prior to the experiment, the cells from one tissue culture flask were divided into wells of a multiwell tissue culture plate (2.0 sq cm/well; Falcon Labware, Oxnard, CA) and allowed to become adherent. The cells were then incubated in serum-free medium with the indicated concentration of 12-HETE, 15-HETE, or arachidonic acid, all in 0.5% v/v ethanol or with 0.5% ethanol control for the experiments. Ethanol at this concentration did not affect [3H]thymidine incorporation into these cells. Each condition was assayed in triplicate.

[3H]Thymidine incorporation was then measured. The test medium was removed, and the cells were washed and incubated with [3H]thymidine (26 Ci/μmol, 12 μCi/ml; New England Nuclear) in fresh complete medium for 40 hr. After washing, the cells were removed from the plates with trypsin (0.125 g/100 ml):EDTA (0.05 g/100 ml), with added unlabeled 0.4 mM thymidine. Chloroacetic acid precipitated material was collected on 0.3-μm Millipore filters (Millipore Corp., Bedford, MA) which were dried and counted in ACS scintillant (Amersham Corp., Arlington Heights, IL).

DNA content was determined according to the fluorescent Hoescht Dye 33258 method of Labarca and Paigen (15) using calf thymus DNA as a standard. DNA content was determined 24 hr after plating (base line) or after an additional 48 hr in complete medium with or without the indicated HETE.

**Statistics.** Results were analyzed by randomized complete block analysis of variance (28). The significance between treatments was then determined by Duncan’s multiple-range test.

**RESULTS**

The synthesis of DNA by SK-N-SH neuroblastoma cells, as measured by [3H]thymidine incorporation, was inhibited by HETEs. In serum-free medium, 12-HETE inhibited [3H]thymidine incorporation at concentrations of 20 μM or above (Table 1) (*p < 0.01*). 15-HETE also inhibited [3H]thymidine incorporation (*p < 0.01*), whereas arachidonic acid had no significant effect at these concentrations. In these experiments, 12-HETE-treated cells incorporated significantly less [3H]thymidine than did 15-HETE-treated cells at 30 and 50 μM. These differences from control did not result from loss of adherent cells by HETE treatment, as cell counts from medium removed prior to the [3H]thymidine incorporation assay showed that less than 1% of the estimated plated cells were nonadherent at all concentrations. Culturing cells for 18 hr in the absence of serum did not affect cell viability. When viability was determined, the incorporation of [3H]thymidine during a subsequent 24 hr in complete medium, 95% of cells cultured in either complete or serum-free medium were labeled. Over 99% of the cells retained the ability to exclude trypan blue dye following 18-hr treatment in the absence of serum with 50 μM 12-HETE or 15-HETE.

As shown in Chart 1, the effect of these HETEs on [3H]thymidine incorporation increased with time of exposure. The degree of inhibition caused by 30 μM 12-HETE or 15-HETE increased from 17.8 and 15.5% at 1 hr to 45.5 and 44.1% at 18 hr, respectively.

In order to confirm that these effects of [3H]thymidine incorporation truly reflected an inhibition of DNA synthesis, the increase in total DNA content over 48 hr was measured. The results are shown in Chart 2. For SK-N-SH cells to grow normally for this period, newborn calf serum was a required component of the medium. In complete medium higher concentrations of HETEs were required to inhibit [3H]thymidine incorporation (data not shown), presumably because of binding of these compounds by serum components. Treatment with 12-HETE produced a 20% inhibition of DNA production (*n = 4, *p < 0.01*), while 15-HETE treatment induced a 14% inhibition (*p < 0.05*). The difference between 12-HETE treatment and 15-HETE treatment was not statistically significant (Chart 2).

**DISCUSSION**

Although HETEs are produced by a variety of human cells and tissues, little is known about their biological function. While 5-
HETE and 5,12-di-HETE are potent chemotactic agents (7). 12-HETE and 15-HETE are much less effective. Yet, they are major metabolites of stimulated platelets (17, 20) and leukocytes (2, 8), respectively. We have demonstrated that these 2 lipoxynase products can inhibit DNA synthesis by neuroblastoma cells in vitro.

Cornwell et al. (4) found an inhibition of aortic smooth muscle proliferation by arachidonic acid at a concentration of 16 µM. Inasmuch as this effect was not blocked by indomethacin, was reversed by antioxidants, and occurred with fatty acids which were lipoxynase but not cyclooxygenase substrates, they proposed that lipoxynase products were responsible. Recently, it has been shown that HPETEs are capable of inhibiting lymphocyte proliferative responses (10), but some of the added HPETEs were converted to HETEs. In our study, inhibition of DNA synthesis was produced in response to HETEs devoid of HPETEs.

While such results suggest no mechanism for the observed inhibition, it probably does not result from a detergent effect, since maximal inhibition required prolonged exposure, was not seen with arachidonic acid, and was significantly different with the 2 positional isomers used. Furthermore, no effect on cell viability by these agents was found.

Studies to date have focused on the role of cyclooxygenase products of arachidonic acid metabolism in tumor growth and metastasis (12, 29). Our results suggests that lipoxynase metabolites of arachidonic acid should also be considered as potential modulators of cellular growth and proliferation.

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