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THE EFFECTS OF HISTAMINE H2 RECEPTOR ANTAGONISTS ON MELANOGENESIS AND CELLULAR PROLIFERATION IN MELANOMA CELLS IN CULTURE

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SUMMARY: B16-C3 murine melanoma, A375P human melanotic melanoma, and C32 human amelanotic melanoma cells were incubated in the presence of (0-4mM) H2-antagonists, ranitidine and cimetidine. Cell proliferation, tyrosinase activity and melanin content were monitored. H2-antagonists stimulated tyrosinase activity and melanin accumulation in B16-C3 cells in a dose- and time-dependent manner. Stimulation of enzyme activity and pigment production was accompanied by inhibition of cellular proliferation in B16-C3 cells. The inhibitory concentration of cimetidine was approximately 2-fold higher than that of ranitidine. H2-antagonists failed to stimulate melanogenesis in A375P or C32 cells, but inhibited cellular proliferation in both cell lines. These results are the first demonstration of H2-antagonist induced phenotypic changes in malignant melanoma cells in vitro, and represent a novel mechanism for the previously described in vivo antitumor effects of these agents. © 1991 Academic Press, Inc.

Tyrosinase (monophenol, 3,4-dihydroxyphenylalanine:oxidoreductase, EC 1.14.18.1) is the rate limiting copper-containing enzyme of melanin biosynthesis. It is thought to catalyze at least three reactions: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (dopa), the oxidation of dopa to dopaquinone, and the conversion of 5,6-dihydroxyindole to melanochrome (1). Stimulation of melanin biosynthesis is usually accompanied by inhibition of cellular proliferation in melanoma cells in culture. Thus, melanogenesis is implicated as a marker of melanoma differentiation (2). Because of poor clinical outcome in metastatic melanoma, a number of investigators have examined the therapeutic potential of a wide variety of compounds and immune modalities on experimental melanoma models. Several reports have shown that histamine H2 receptor antagonists alone or in combination with another agent can induce melanoma remission in humans and animals in vivo (3-10). H2-antagonist induced tumor regression has also been reported in humans with gastric carcinoma (11), Kaposi's sarcoma (12), bronchogenic carcinoma (13), and in mice with lymphoma or fibrosarcoma (14), and lung carcinoma (15). The antitumor effect of H2-antagonists in these studies has been attributed to immunomodulation via inhibition of suppressor lymphocyte activity. H2 receptors have been demonstrated in melanoma cells as well as in suppressor lymphocytes. Suppressor cell activity has been shown to be inhibited by H2 receptor antagonists, but direct effects of H2-antagonists on melanogenesis or tumor cell

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proliferation have not been reported. A few studies have shown that histamine stimulates cAMP accumulation in vitro and that H2-antagonists attenuate this effect in melanocytes and melanoma cells, but tyrosinase activity, melanin accumulation or proliferation rate have not been linked to the variations in cAMP levels in these cells (16). We therefore undertook the following studies to characterize the effects of the histamine H2 receptor antagonists, ranitidine and cimetidine, on melanogenesis and cellular proliferation in murine and human melanoma cells in culture in an attempt to explain their in vivo antitumor effects.

MATERIALS AND METHODS

Chemicals: Ranitidine was a gift from Glaxo Inc., and cimetidine was a gift from Smith-French and Kline Laboratories. Minimal Essential Medium (MEM) with Earle's salts was obtained from Gibco Laboratories, Grand Island, NY. [^{3}H]L-tyrosine (specific activity 59.0 Ci/mM) was obtained from New England Nuclear, Boston, MA. All chemicals used were of highest purity available.

Cell Cultures: B16-C3 mouse melanoma cells were a gift from Dr. Ellis L. Kline (Clemson University, SC). A375P cells (17) were obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). C32 cells were obtained from American Type Culture Collection, Maryland. Cells were maintained in a 37°C, humidified 5% CO2 incubator, covered with MEM supplemented with 10% heat inactivated calf serum (B16-C3 cells) or fetal calf serum (A375P and C32 cells). Cells were removed for passage with EDTA (1:5000). Exponentially proliferating cells were seeded at a density of $1x10^4$ cells/cm^2 and allowed to attach for 12-18 hours. Cultures were then incubated with fresh medium supplemented without or with ranitidine (0-4 mM) or cimetidine (0-4mM).

Tyrosinase assay: Tyrosinase activity in whole cell sonicates was determined as reported by Pomerantz (18). Briefly, culture plates were rinsed with phosphate buffered saline (PBS) twice, and harvested in potassium phosphate buffer (80 mM, pH 7.0), and frozen at -20°C until assayed. Samples were thawed and disrupted at 4°C with two 10-second sonication bursts with a microtip probe. The assay reaction mixture contained 0.4 μM L-tyrosine, 0.04 μM L-dopa, 16.0 μM potassium phosphate buffer pH 7.0, and 0.32 μCi [^{3}H]L-tyrosine, and 0.2 ml cell sonicate (final volume 0.4 ml). This mixture was incubated for 1 hour in 37°C water bath. Reactions were terminated by the addition of 0.5 ml ice-cold trichloroacetic acid (TCA) (10%, w/v). Unreacted [^{3}H]L-tyrosine was removed by adding 0.7 ml activated charcoal suspension (100 mg/ml Norit-a, Fisher) and centrifugation. A 100 μl aliquot of supernatant was counted by liquid scintillation spectroscopy using Aquosol II as a scintillant. Protein concentrations were determined by the method of Lowry et al. (19). Results were expressed as cpm or dpm per μg of cellular protein.

Melanin assay: Melanin concentration was determined by a modified method of Huberman et al. (20). Briefly, cell monolayers were harvested in PBS using rubber policemen. Cellular material was precipitated with TCA (5%), washed with ethanol-ether (3:1 v/v) and dried. The residue was dissolved in 0.85 N potassium hydroxide by heating to 95°C for 15 minutes. Optical density measurements were performed at 400 nm. A standard curve was generated using known amounts of melanin. Results were expressed as μg of melanin per mg of cellular protein.

Assessment of Proliferation: Cells at exponential growth phase were plated ($0.5x10^2$ cells/cm^2) in gridded petri dishes for serial cell counts. Growth rate was determined visually by monitoring the increase in the number of cells in selected groups every 24 hours.

RESULTS

Effects of H2-antagonists on tyrosinase activity: Rapidly proliferating B16-C3 cells were incubated in the presence of 0-4mM ranitidine or cimetidine for 24 hours. Ranitidine stimulated the tyrosinase activity in a dose dependent manner up to 4mM (Figure 1). Stimulation was maximal at 3mM, and half-maximal at 1mM. Concentrations beyond 4mM resulted in cell degeneration noticeable by vacuole formation and cell detachment. Cimetidine stimulated the tyrosinase activity in B16-C3 cells at a comparable concentration range. Induction of the enzyme

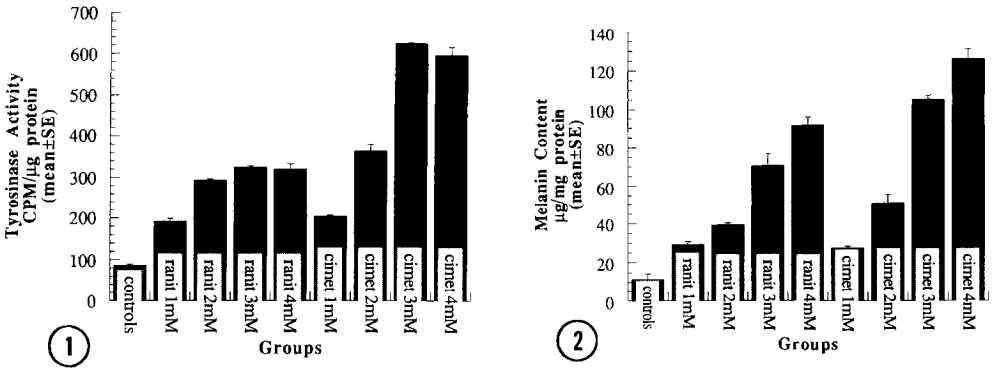

Figure 1. Dose response of ranitidine and cimetidine effects on tyrosinase activity in cultured B16-C3 mouse melanoma cells. Rapidly proliferating cells were treated with 0-4mM concentrations of ranitidine (ranit) or cimetidine (cimet) for 24 hours, and tyrosinase activity was determined as detailed in the methods section. Each group represents the mean ± SE from a representative experiment.

Figure 2. Dose response of ranitidine and cimetidine effects on melanin accumulation in cultured B16-C3 murine melanoma cells. Rapidly proliferating cells were treated with 0-4mM concentrations of ranitidine (ranit) or cimetidine (cimet) for 24 hours, and melanin accumulation was determined as detailed in the methods section. Each group represents the mean ± SE from a representative experiment.

activity with cimetidine was more potent than with ranitidine. With H2-antagonist treatment, enzyme activity increased in a time-dependent manner over the duration of the study (48 hours). At 48 hours, tyrosinase activity was 2029±24 dpm/µg protein in treated cultures vs. 333±11 in controls. H2-antagonists failed to stimulate the tyrosinase activity in A375P or C32 cells (data not shown).

Effects of H2-antagonists on melanin biosynthesis: The dose-dependent increase of tyrosinase activity in H2-antagonist induced B16-C3 cells was paralleled by melanin accumulation (Figure 2). Melanin levels in ranitidine treated cultures were 3-fold higher than controls at 1mM, and 7-fold higher at 4mM. Cimetidine had similar effects on melanogenesis in B16-C3 cells. Melanin accumulation was higher in cimetidine groups than ranitidine groups at most concentrations tested. Time course studies revealed that melanin accumulation was increased 2.5-fold by 12 hours. At 48 hours the cells were heavily pigmented macroscopically, and melanin content was 44±1.4 µg/mg protein in ranitidine treated cultures vs. 7.3±0.3 in controls. H2-antagonists failed to stimulate melanin accumulation in A375P or C32 cells (data not shown).

Effects of H2-antagonists on cellular proliferation: B16-C3 cells proliferated exponentially after a lag period of about 12 hours. The doubling time was approximately 24 hours during the exponential growth phase. H2-antagonists suppressed the cellular proliferation within 24 hours in a dose dependent manner when added to the culture medium of rapidly proliferating B16-C3 cells (Figure 3). While untreated cultures continued to proliferate rapidly over the course of the study (144 hours), the presence of 2mM ranitidine in the medium completely inhibited the cellular proliferation within 24 hours. Inhibitory concentrations for cimetidine were approximately 2-fold higher than that for ranitidine. Cimetidine at 2mM concentration slowed the proliferation rate significantly; total inhibition was achieved at 4mM. Loss of cytoplasmic processes was

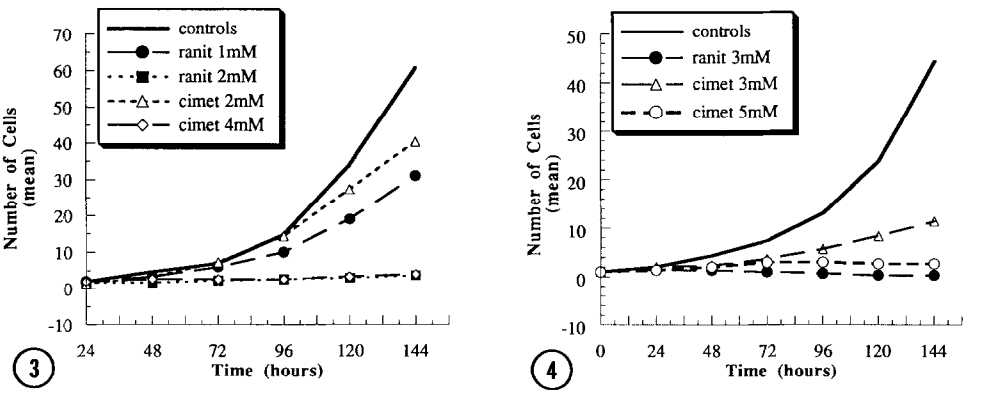

Figure 3. Effect of varying concentrations of ranitidine and cimetidine on B16-C3 cell proliferation. Sparse, rapidly proliferating cells were incubated without or with ranitidine (ranit, 1mM or 2mM) or cimetidine (cimet, 2mM or 4mM). Three pairs of cells were identified in each plate and proliferation rates were determined by serial assessment of the number of daughter cells. Each point represents the mean from a representative experiment.

Figure 4. Effect of ranitidine and cimetidine on A375P human melanotic melanoma cell proliferation. Sparse, rapidly proliferating A375P cells were incubated without or with ranitidine (ranit, 3mM) or cimetidine (cimet, 3mM or 5mM). Three pairs of cells were identified in each plate and proliferation rates were determined by serial assessment of the cell number. Each point represents the mean from a representative experiment.

noticeable in treated cells. The doubling period of untreated A375P human melanotic melanoma cells was 26 hours. Incubations with ranitidine or cimetidine resulted in complete inhibition of cellular proliferation in A375P cells in a dose-dependent manner within 48-72 hours (Figure 4). C32 human amelanotic melanoma cells had a doubling period of 38 hours. H2-antagonists inhibited the proliferation of C32 cells in a similar manner to A375P cells (data not shown). Antiproliferative concentrations of cimetidine in A375P and C32 cells were approximately 2-fold higher than those for ranitidine.

DISCUSSION

The low yield of current surgical and chemotherapeutic approaches has resulted in a search for alternate modalities which might influence the outcome in malignant melanoma. An approach to tumor immunotherapy in patients and in experimental systems has involved attempts at selective pharmacological inactivation of the host suppressor cells which undermine an effective anti-tumor response. One characteristic of suppressor cells that offers the possibility of pharmacological manipulation is the presence of histamine H2 receptors (21). Histamine is a potent activator of suppressor cells in man and in animals. This effect of histamine is specifically suppressed by histamine H2 receptor antagonists, but not by H1-antagonists (22).

Cimetidine-containing regimens have been reported to induce remission in patients with metastatic melanoma (3,8,10). There is also a case report of a patient who developed complete regression of metastatic melanoma on oral ranitidine prescribed for nonspecific gastrointestinal symptoms (7). Cimetidine has been shown to inhibit tumor proliferation, and to inhibit the development of metastasis in a variety of carcinomas in mice (14,15). Prolongation of survival has been associated with inactivation of suppressor lymphocytes. Cimetidine-induced enhancement of

the immune response is postulated to be due to inhibition of suppressor cell activity in response to tumor challenge. H2 receptor antagonists have also been shown to augment natural killer cell activity in normal subjects and in patients with advanced melanoma and colorectal carcinoma (23). Proliferation of Cloudman melanoma cells in mice has been shown to be accelerated by histamine treatment, and blocked by μM concentrations of cimetidine in vivo (24).

In all these reports, the effects of H2-antagonists have been attributed to their action on immune system cells rather than to any direct antiproliferative properties on tumor cells. Stimulation of H2 receptors with histamine has resulted in significant elevations in cAMP levels in human melanoma cells. The cAMP response is negated by the addition of μM concentrations of ranitidine or cimetidine to the culture medium (16). In contrast to other conditions where variations in cAMP levels are shown to affect cellular proliferation and metastatic potential in melanoma cells (25,26), H2 receptor-mediated cAMP accumulation or blockade of this effect with H2-antagonist has never been reported to be linked to any phenotypic changes in melanoma cells. Thus, no quantifiable changes in melanin content, tyrosinase activity or proliferative characteristics have been demonstrated in melanoma cells in response to H2-antagonists.

Our results demonstrate that mM concentrations of H2-antagonists stimulate tyrosinase activity, and melanin accumulation, and inhibit cellular proliferation in B16-C3 cells. In contrast to earlier beliefs, these findings suggest that H2-antagonists may exert direct antitumor action and induce melanogenesis in melanoma cells. This represents a novel mechanism for the previously described in vivo antitumor effects of these agents. Antiproliferative action of H2-antagonists is accompanied by stimulation of melanogenesis in B16-C3, but not in A375P or C32 cells. The precise mechanism of action of H2-antagonists on melanogenesis and cellular proliferation in melanoma cells is not known. Our observation that H2-antagonists inhibit proliferation without inducing melanogenesis implies that proliferation and melanogenesis can be independent of each other in some melanoma cells. These results are the first demonstration of direct effects of H2-antagonists on melanogenesis and cellular proliferation in murine and human melanoma cells. Recent clinical reports demonstrating possible effectiveness of H2-antagonists in gastric carcinoma and in HIV-related Kaposi's sarcoma implies broader potential applications for this class of agents.

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