Suppression of Melanoma Cell Proliferation by Histidine Decarboxylase Specific Antisense Oligonucleotides

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Histidine decarboxylase (HDC) is expressed by the cells of melanoma, in which the histamine content tends to be relatively high. This study shows that elevated expression of HDC was found by western blot analysis of primary and metastatic melanoma tissue using a polyclonal HDC specific antibody. The specificity of anti-HDC antibody was confirmed by inhibition of HDC translation (i.e., immunopositivity) in melanoma cells by HDC-specific antisense oligonucleotide. Moreover, the decrease in proliferation caused by HDC antisense oligonucleotides indicates considerable functional relevance of histamine synthesis in melanoma growth and suggests a possible *in situ* application of specific antisense oligonucleotides for HDC in melanoma therapy. *J Invest Dermatol 117:151–153, 2001*

According to recent ideas, the production of growth factors by melanoma cells may provide an escape from exogenous mechanisms regulating the proliferation of normal cells (Moretti *et al*, 1999). Histamine, one of the biogenic amines with general physiologic importance, occurs abundantly in tumor tissue, and high gene expression of L-histidine decarboxylase (HDC) has been recently described in melanoma tissue and cell lines (Haak-Frendscho *et al*, 2000). This study shows a high HDC expression in clinical samples from 25 patients suffering from melanoma. Striking antiproliferative effect of HDC-specific antisense oligonucleotides (ASO) demonstrated under *in vitro* conditions suggests a possible successful alternative way for the local human therapy of melanoma.

MATERIALS AND METHODS

Patients and tissue samples Thirty-five human tissue samples were studied. The tissue biopsies were removed by surgical excision from 28 patients. The samples were frozen at –80°C for western blotting.

The sex and age distribution of 28 patients was as follows: 15 females (between 28 and 86 y) and 13 males (from 34 to 81 y). The examined samples were: three dysplastic naevi (two females, one male), 20 melanomas (melanoma at different Clark stages and Breslaw index; 12 females, eight males), five metastatic melanoma lesions (cutaneous or lymph node; three males, two females), and 10 samples from skin regions surrounding the primary cutaneous melanoma (five males and five females).

Melanoma cell lines Primary melanomas: WM35 and WM983/B (growing preferentially vertically and horizontally, respectively). Metastatic lines: HT168 (metastatic xenograft, previously generated subcutaneous *in vivo* passaging of A2058 cells in IS CBA/Ca mice) cell line with low liver metastatic capacity, and M1/15 with high liver metastatic capacity.

Manuscript received December 21, 2000; revised March 19, 2001; accepted for publication April 9, 2001.

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HDC western blotting and immunostaining Primary polyclonal antibodies against human HDC protein were produced in chicken (IgY) (Haak-Frendscho *et al*, 2000). Antibodies were prepared using the amino acid sequence 318–325 (VKDKYKLQ) of the human HDC and used after affinity purification. Specificity of anti-HDC antibodies is demonstrated in **Fig 1**, showing that absorbance with increasing amount of immunizing fusion construct removes the 55 kDa (HDC) band.

The cells were lyzed and homogenized in a buffer containing 10 mM Tris HCl pH 8.0, 1% Triton-X, 0.5 mM EGTA, 10 μg leupeptin per ml, 5 mM NaF, 50 mM Na-othovanadate, 25 mM PMSF. Fifteen micrograms lysate was loaded on the denaturing polyacrylamide gel (10% running gel and 5% stacking gel, 110 V, 20 mA, 2 h). After blotting (Hoefer instrument, Amersham Immobilon-P paper, Tris-HCl, glycine, methanol buffer, 370 mA, 45 min), the membrane was gently shaken in a blocking solution [5% low-fat milk powder in phosphate buffered saline (Sigma, Budapest, Hungary) + 0.1% Tween 20] for 1 h at room temperature. HDC band was revealed by subsequent treatment with anti-HDC antibody (40 ng per ml for 1 h). HRPO-labeled antichicken IgY secondary antibody (Promega, Madison, WI, 100 ng per ml) was then used for 30 min and visualized with an ECL (Amersham, Bucks) Western blotting kit. For immunohistochemistry of melanoma cell lines, monolayers were fixed in acetone (20°C, 10 min), then stained by chicken anti-HDC antibody (10 μg per ml, overnight, 4°C). After three washes with ice-cold PBS, FITC labeled rabbit antichicken IgY secondary antibody (0.5 μg per ml, 20°C, 3 h) was applied. Cell nuclei were stained with 1.5 μg per ml propidium iodine (Sigma). After three washes, the cells were examined with a confocal microscope (Bio-Rad, Budapest, Hungary).

HDC-specific ASO ASO were selected based on the sequence data of HDC cDNA (Accession NM_002112). Three HDC ASO – HGT AGA CGG TCA TGG ACH (cDNA nt. 71–87), HCT CGG GTT CCC TCT ACH (cDNA nt.90–105), HHT ATA AAA CTT AGA GAC (cDNA nt.1260–1275) – were tried in previous optimization experiments (not shown) and the one corresponding to the positions of cDNA nt. 1260–1275 was used in the experiments.

Cell proliferation Melanoma cells (10^5 per ml) were incubated on 24-well plates in the presence of HDC ASO (30 μg per ml) and 20 μg per ml DNA uptake stimulating factor (DUSF; Tóth *et al*, 1998) in the medium. Nonsense oligonucleotide mixture and DUSF were used as control. After 48 and 96 h, the cells were washed with PBS-EDTA

(0.01 M) and counted. Uptake and catabolism of ASO in melanoma cells and other cell lines had been controlled as in earlier experiments (Radvány *et al*, 2000). Briefly, ^{3}H-labeled antisense oligonucleotides were mixed with HDC-ASO in the presence of increasing amounts of unlabeled ASO (both antisense and nonsense). After incubation and washings, the uptake and stability of ASO were tested. These experiments showed the specific inhibition of HDC antisense ASO by ''cold'' specific, but not by ''cold'' nonsense oligonucleotides. Moreover, the stability of HDC ASO were confirmed by the activity of tritiated ASO decreasing by less than 2% over a further 72 h. All experiments were performed in triplicates.

Statistical analysis was performed by Student t analysis and by one way ANOVA.

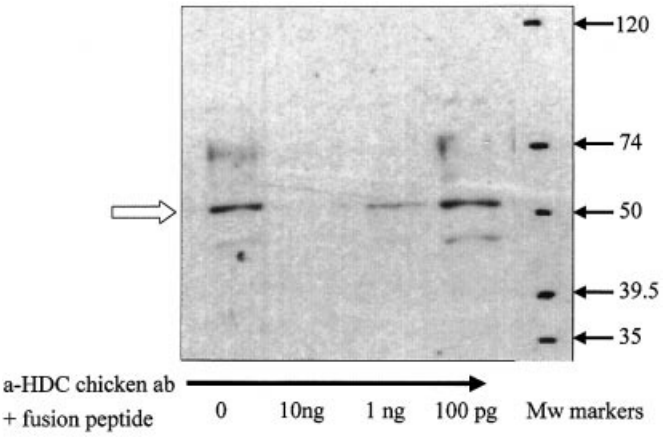

Figure 1. Western immunoblotting of polyclonal anti-HDC chicken antibody with a lysate from melanoma cell line WM35. The affinity purified antibody had been absorbed with increasing amounts of immunogenic fusion protein containing the specific immunogenic peptide (VKDKYKLQ) sequence.

RESULTS AND DISCUSSION

Table I shows the frequency of HDC protein in melanoma biopsies. Strong expression of HDC in both primary and metastatic

Table I. HDC immunopositivity (western immunoblot) of tissue specimen from patients with primary melanoma, metastatic tissues, and naevi and from normal skin samples (for details see *Materials and Methods*)

Tissue specimen	0	+ ve
Primary melanoma[a]	1	19
Metastasis[b]	0	5
Dysplastic nevi[c]	1	2
Normal skin[d]	8	2

[a]Twelve females, 8 males; [b]2 females, 3 males; [c]2 females, 1 male; [d]5 females, 5 males.

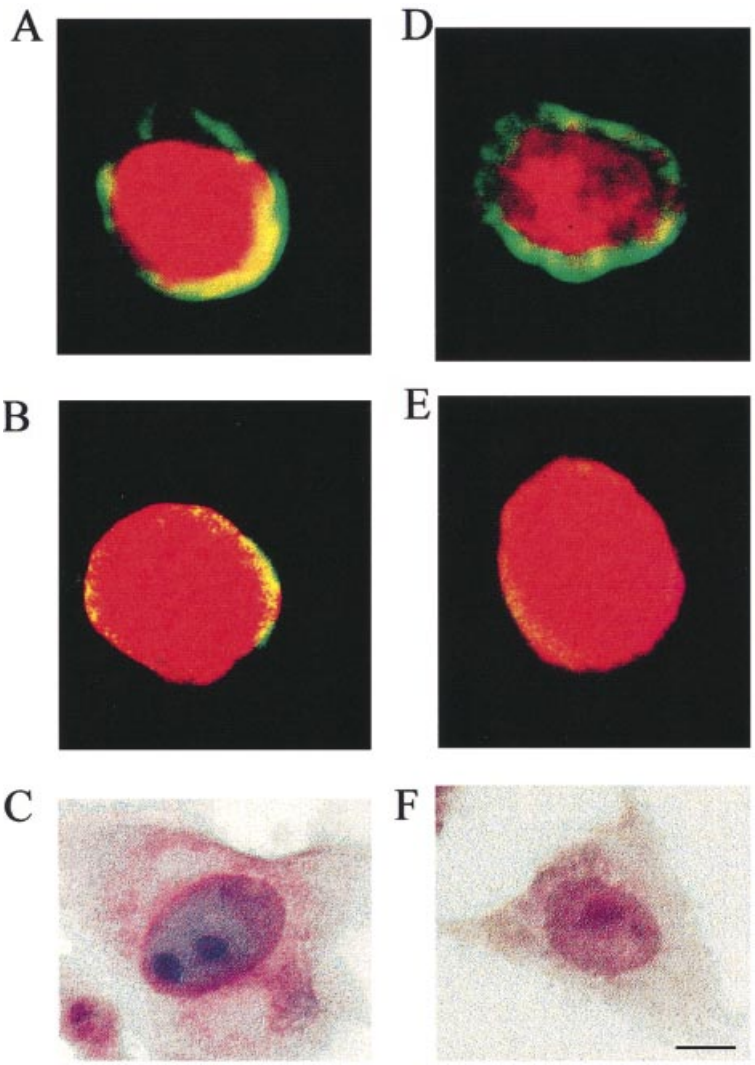

Figure 2. Effect of HDC antisense oligonucleotides on HDC immunopositivity of human melanoma cells. (*A*, *B*) WM983/B, (*D*, *E*) HT168/91 human melanoma cells in the presence of mixture of nonsense oligonucleotides (*A*, *D*) and HDC antisense oligonucleotide (*B*, *E*). *Scale bar*: (*A*, *B*, *D*, *F*) 5 μM; (*C*, *F*) 10 μM.

melanoma and low frequency in naevi and normal skin with about 25–10-fold lower expression confirms our earlier data (Haak-Frendscho *et al*, 2000) concerning the presence of HDC mRNA and protein in melanoma cell lines and tissues and their absence in normal human melanocytes.

HDC-specific and nonsense 18-mer ASO were taken up by both the primary (WM 983/B, WM 35) and the metastatic (HT168/91, M1/15) human melanoma cell lines, as controlled by uptake of ^{3}H-labeled nucleotides (not shown).

Specific ASO completely abolished (**Figs 2*B*, *E***) the immunostaining (i.e., translation) of HDC in WM983/B (A-C) and HT168/91 (D-F), confirming the specificity of the anti-HDC antibody. Mixture of nonsense 18-mer ASO (**Figs 2*A*, *D***) had no effect on HDC immunoreactivity.

As a functional consequence of the inhibition of HDC protein synthesis, specific antisense oligonucleotide strongly (> 50%) decreased the proliferation rate of both WM938/B and HT168/91 human melanoma cell cultures by 48–96 h (**Fig 3**), whereas no effects of the nonsense ASO were detected. Similar effects were found with the other two melanoma cell lines WM35 and M1/15 (not shown).

Following the first observation of the presence of histamine in rapidly growing tissues (Kahlson *et al*, 1963), numerous experiments in tumor-bearing animals have suggested that high HDC activity is of fundamental importance for tumor development (Cricco *et al*, 1994). Under various tumor conditions, endogenous histamine may act as an autocrine growth factor, influencing cell proliferation via H2 histamine receptors (Burtin *et al*, 1988; Bolton *et al*, 2000). A more indirect way of influencing melanoma growth by locally produced histamine is by its suppression of the production of IFN-γ by surrounding immune cells (e.g., T lymphocytes and natural killer cells; Horváth *et al*, 1999), which effectively impairs their antitumor activity and contributes to escape of melanoma cells from immunosurveillance. Interestingly, IFN-γ in turn decreases HDC expression (Heninger *et al*, 2000). These reciprocal effects reflect a highly complex regulatory network acting in the close microenvironment of melanoma cells. Because histamine influences *de novo* angiogenesis locally, it acts as a further form of tumor surveillance that is enhanced by locally produced histamine (Rizzo and DeFouw, 1996).

Our data demonstrate that HDC-specific antisense oligonucleotide substantially decreases the expression of HDC in melanoma cell lines, with simultaneous inhibition of their proliferation. These data are promising because recently increasing attention has been drawn toward locally therapeutic application of antisense oligonucleotides by Akhtar and Agrawal (1997). An 18-mer phosphorothioated ASO complementary to *bcl*-2, administered at 5 mg per kg per d for 21 d to severely compromised, immunodeficient (SCID) mice inoculated with follicular lymphoma cells eliminated lymphoma cells in six of six mice (Wagner, 1997). Moreover, the *in vivo* transduction of prostate cancer cells with MMTV-antisense c-myc retroviruses reduced tumor growth by suppressing c-myc, resulting in the downregulation of bcl-2 protein (Steiner *et al*, 1998). A similar successful approach has been described in the course of therapy for tumors of the central nervous system by Chung and Chiocca (1998). Free access to genomic data and EST libraries of melanoma cells provide a greater chance that not only traditional antihistamines, such as H2 histamine antagonists (Bolton *et al*, 2000), but also antisense oligonucleotides targeting HDC translation, may also soon become candidates for a locally applicable, *in situ* therapy for melanoma.

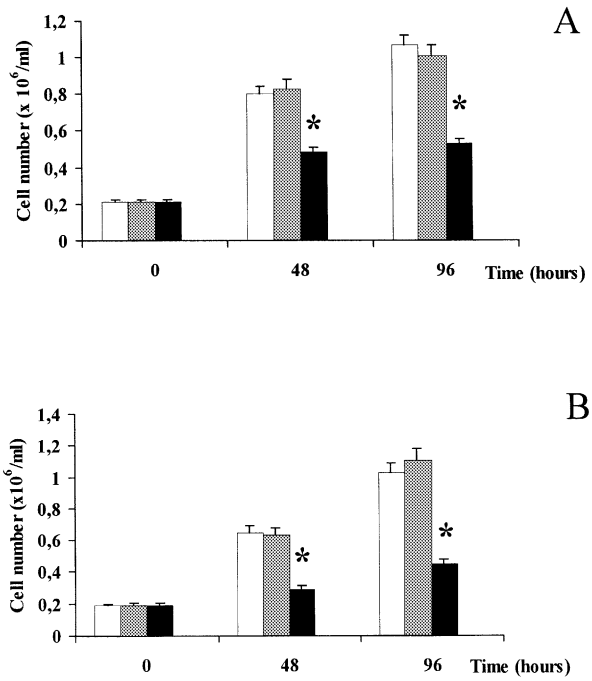

Figure 3. The effect of an 18mer HDC-ASO on the proliferation of WM938/B and HT168/91 human melanoma cells. (*A*) WM938/B; (*B*) HT168/91. Control: open columns, nonsense oligonucleotide mixture: stripped columns, HDC ASO: black columns. $N = 4$, mean ± SEM is shown. *$p < 0.01$.

The authors are grateful to Professor Denys Wheatley and Dr. Erna Pap for critical reading of the manuscript. This work was supported by Hungarian-Argentinian TéT contract (ARG-9/98), OTKA021175, 031887, ETT 300/2000, FKFP 1075/97, SOTE 1999.

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