SPANDIDOS PUBLICATIONS

Real-time RT-PCR analysis of human histidine decarboxylase, a new marker for several types of leukemia and cancer

ESTHER MELGAREJO, MIGUEL ÁNGEL MEDINA, JOSÉ CARLOS PAZ[1], FRANCISCA SÁNCHEZ-JIMÉNEZ and JOSÉ LUIS URDIALES

Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of Málaga, E-29071 Málaga, Spain

Received February 25, 2005; Accepted May 30, 2005

Abstract. Histamine is involved in different physiological and pathological responses, such as immune response, gastric acid secretion or neurotransmission, as either angiogenesis or cancer. Histidine decarboxylase (HDC) catalyzes the formation of histamine from histidine. HDC has been suggested as a new marker for neuroendocrine differentiation, inflammatory pathologies and several leukemia and highly malignant forms of cancer, such as melanoma and small cell lung carcinoma. In the present work, we describe the use of Syber Green-based quantitative real-time RT-PCR to determine the expression of histidine decarboxylase in human cells and tissue. As an internal control, glyceraldehyde 3-phosphate dehydrogenase was also amplified. The linear dynamic range of the assay covered 4 orders of magnitude for HDC amplification. The detection limit was 0.1 ng of total RNA extracted from HMC-1 cells. This method is simple, rapid, sensitive, and quantitative, and allows for the specific identification of cells and tissue expressing HDC, stressing its potential diagnostic usefulness in malignancies in which HDC is described as a new marker.

Introduction

The biogenic amine histamine plays a pivotal role in several physiological responses, including immune response, gastric acid secretion and neurotransmission, among others (1). L-histidine decarboxylase (HDC) is the only enzyme that catalyzes the formation of histamine from L-histidine, which is expressed in the cell types involved in histamine production and secretion, including mast cells, monocytes/macrophages, basophils, enterochromaffin-like cells, and histaminergic neurons (1). However, histamine is also involved in several pathological disorders, including angiogenesis and other inflammation-related processes, as well as cancer (1-3). HDC has previously been shown to be overexpressed in human melanoma (4), small cell lung carcinoma (5), several types of neuroendocrine tumors (6,7) and chondrocytes of arthritic cartilage (8). It is also well documented that HDC is overexpressed in myelogenous leukemia lymphoblasts, mastocytomas and severe mastocytosis (9-11). Therefore, HDC has been suggested as a new marker for neuroendocrine differentiation (6) and as a good diagnostic tool for inflammatory pathologies and highly malignant tumors (4-8). Traditionally, HDC expression is detected by Northern or Western blot analysis, RT-PCR, or immunohistochemistry (4-8,12,13). However, a reliable and automatizable quantitative method to determine HDC expression for use in the clinical analysis laboratory is urgently required.

The aim of the present study was to develop a protocol for the quantitative determination of HDC mRNA transcripts in human cells and tissue. As a reference source we used human HMC-1 mast cells, a cell line expressing high levels of HDC as revealed by Northern blotting. To fulfill our purpose, we next developed a real-time RT-PCR procedure. Unlike endpoint RT-PCR, real-time quantification is defined by C_T (threshold cycle number) at a fixed threshold where PCR amplification is still in the exponential phase and the reaction components are not limiting gene amplification. We evaluated the pre-analytical conditions required for the use of HDC mRNA measurements as a diganostic tool in the clinical analysis laboratory. We analyzed relative standard curves (14,15). Additionally, we carried out parallel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene expression determination, as a renormalization strategy. Finally, we tested the performance of the procedure by using samples of total RNA extracted from the human KU812F myelogenous leukemia lymphoblast cell line (expressing HDC) and two human tumor cell lines that do not express HDC, namely the HT-1080 fibrosarcoma and MDA-MB231 breast cancer cell lines.

Correspondence to: Dr J.L. Urdiales, Department of Molecular Biology and Biochemistry, Faculty of Sciences, University of Málaga, E-29071 Málaga, Spain
E-mail: jlurdial@uma.es

Present address: [1]Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, Avenida Diagonal 645, E-08028 Barcelona, Spain

Key words: histidine decarboxylase, real-time RT-PCR, Syber green, human mast cells, human myelogenous leukemia, breast cancer, fibrosarcoma

Table I. List of primers used for HDC and GAPDH amplification.

	HDC	GAPDH
Gene Bank Access No.	M60445	BC004109
Forward primer (5'-3'	GCCCAAGGGAGATGATGGAG	TGCACCACCAACTGCTTAGC
Reverse primer (5'-3')	GGCTGGATGCCCAGGTGAAT	GGCTGGACTGTGGTCATGAG
Amplicon size (bp)	341	88

Materials and methods

Cell culture. Human HMC-1 mast cells were kindly supplied by Dr J.H. Butterfield (Mayo Clinic, Rochester, MN, USA) and maintained in Iscove's medium supplemented with 10% calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml amphoterycin, iron supplement and 1.2 mM α-thioglycerol. These cells grow in suspension and their density was maintained at 0.1-1.5x10^6 cells/ml.

Human KU812F myelogenous leukemia lymphoblast cells were supplied by the European Collection of Animal Cell Cultures and were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 1.25 μg/ml amphoterycin. These cells grow in suspension and their density was maintained at 3-9x10^5 cells/ml.

Human MDA-MB231 breast cancer and HT-1080 fibrosarcoma cells were obtained from the American Type Culture Collection. MDA-MB231 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 1.25 μg/ml amphoterycin. These adherent cells were sub-cultured twice a week at a 1/4 split ratio. HT-1080 cells were maintained in DMEM (containing glucose at 4.5 g/l) medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 1.25 μg/ml amphoterycin. These adherent cells were sub-cultured twice a week at a 1/5 split ratio.

RNA isolation. Total RNA from the different cells tested was extracted using the RNeasy mini kit (Qiagen) or the GenElute Mammalian Total RNA miniprep kit (Sigma). RNA was quantified and the quality tested by photometric measurement. We only used highly purified RNA (A_{260}/A_{280}>1.95).

Real-time RT-PCR. Oligonucleotide primers for the amplification were designed using Amplify 1.2 software (16). The selected specific and optimal sequences, shown in Table I, were chosen to prevent amplification of genomic DNA. According to the suggestion to abandon the one enzyme/one tube protocols for real-time RT-PCR (17), we carried out two step RT-PCR. Reverse transcriptions were performed for 1 h at 42˚C in a final volume of 25 μl, containing 5 μg of total RNA (previously denaturated for 10 min at 65˚C), 1.3 mM dNTPs, 800 nM antisense primer and 200 units of MMLV-reverse transcriptase (Promega). PCRs were carried out in a final volume of 25 μl, containing 1 μM of both primers, 1x Syber Green supermix (Bio-Rad), and variable amounts of RT products. Thermal cycling was peformed in an iCycler iQ (Bio-Rad), evaluated as a very impressive instrument in a recent review on real-time RT-PCR for mRNA quantification (17). Data were treated with the accompanying software iCycler iQ Optical System Software 3.0a. The program profile used for HDC amplification was: 95˚C for 3 min and 50 cycles of denaturation for 20 seconds at 95˚C, and annealing for 15 seconds at 58˚C and extension for 30 seconds at 72˚C. The program profile used for GAPDH was 94˚C for 3 min followed by 30 cycles of denaturation, annealing and extension for 30 sec each at 94˚C, 67.5˚C and 72˚C, respectively.

Results

HDC expression in HMC-1 cells. RNA isolated from HMC-1 cells, as described above, was used to detect HDC mRNA by Northern blot analysis. As a probe, the RT-PCR product obtained with the oligos described in Table I and confirmed by sequencing was used. Fig. 1 shows that there was a high expression level of HDC in HMC-1. As previously reported, two HDC mRNA species were observed, which were supposed to be generated by alternative splicing (10,18).

Development af a quantitative assay for HDC mRNA. The characteristics of the assay were evaluated according to the guidelines for the validation of analytical procedures (19,20). From amplification curves for HDC and GAPDH (Fig. 2A and B), C_T values at a fixed threshold of relative fluorescence were determined. Calibration curves were constructed by plotting C_T values as a function of log of total RNA, assuming that RNA targets were reversed, transcribed, and subsequently amplified with similar efficiency (Fig. 2C and D) (17). Amplification efficiencies were calculated from the slopes of linear regression curves according to the expression $E=10^{(-1/slope)}$, and were 2.620±0.176 for HDC and 2.110±0.161 for GAPDH. The linear dynamic range of the assay covered over 4 orders of magnitude for HDC and 5 orders of magnitude for GAPDH. Intra-assay CVs were <3% for HDC and <4% for GAPDH. Analysis of the melting curve profiles confirmed the specific accumulation of the amplification products (Fig. 3A and B), with a single melting point for HDC (Tm= 89.0±0.2˚C) and a single melting point for GAPDH (Tm=83.0±0.2˚C). The

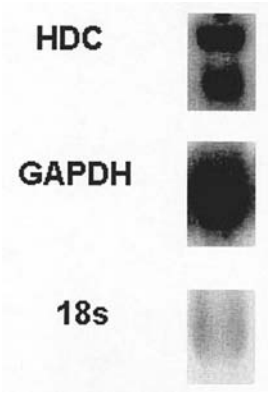

Figure 1. Expression of HDC mRNA species in HMC-1 cells as detected by Northern blot analysis. As internal controls, GAPDH signal after hybridization with a specific probe, and 18S rRNA in gel as detected with ethidium bromide, are shown.

detection limit based on visual evaluation was 0.1 ng of total RNA extracted from HMC-1 cells.

Expression of HDC mRNA in other cell lines. The experimental procedure and the analytical model were therefore applied to test the performance of the assay in situations of clinical relevance. The goal was to test whether the procedure discriminates samples expressing or not expressing HDC. Total RNA samples extracted from a human tumor cell line expressing HDC (basophilic KU812F myelogenous leukemia lymphoblast cells), and two human tumor cell lines that do not express HDC, namely the HT-1080 fibrosarcoma and MDA-MB231 breast cancer cell lines, were used, along with samples from HMC-1, as internal positive controls. As observed in the melting curve profiles, there was a specific amplification of HDC in samples from

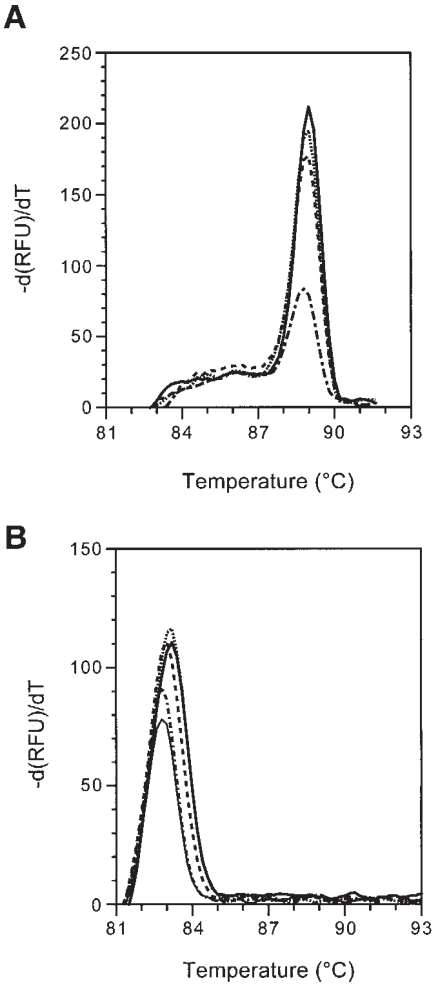

Figure 3. Melting curves for the whole dilution series used for HDC (A) and GAPDH (B). A unique maximum (melting point) is a signal for specific accumulation of the amplification product of each target.

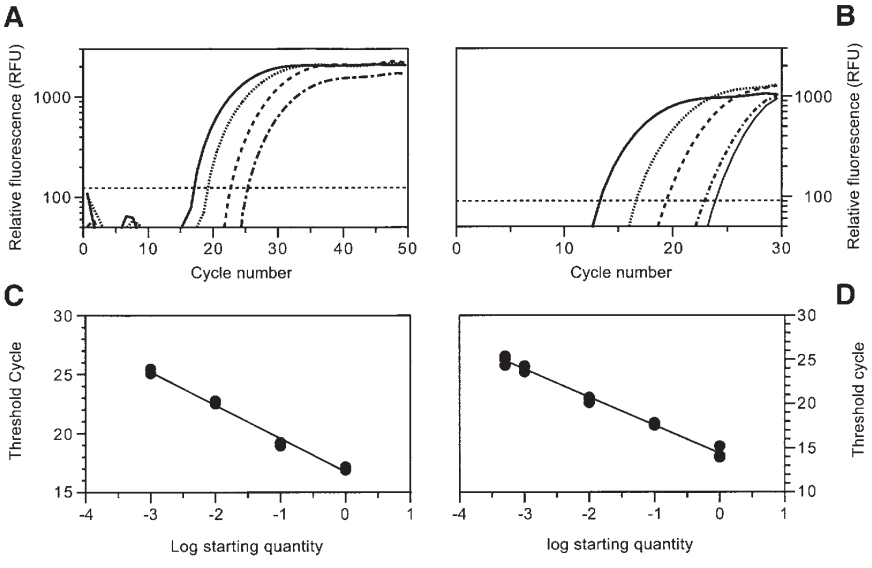

Figure 2. Specific amplification of HDC and GAPDH from human HMC-1 mast cells. Amplification curves for HDC (A) and GAPDH (B) from different dilutions of HMC-1 cell samples. The amplification of HDC and GAPDH were carried out from 1 (bold line), 0.1 (dotted line), 0.01 (dashed line), 0.001 (dot-dashed line) and 0.0005 μg of RNA (thin line). Calibration curves for HDC (C) and GAPDH (D) used for determination of PCR efficiencies.

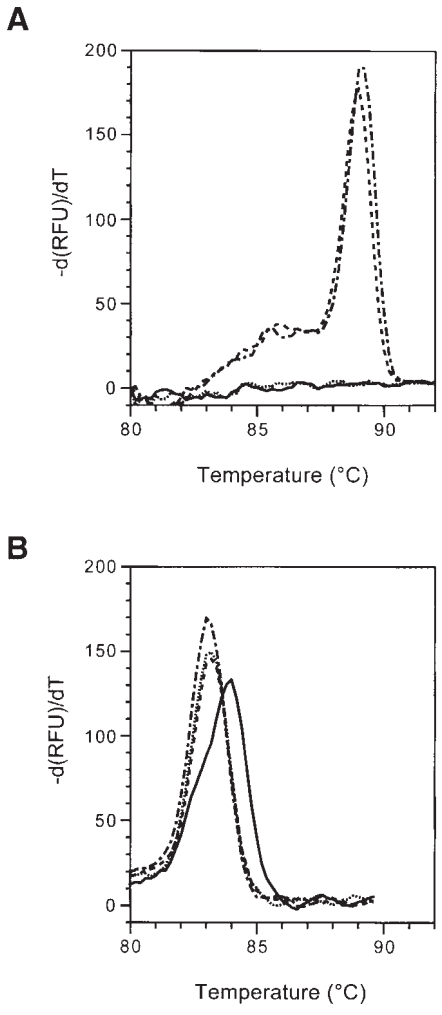

Figure 4. Melting curves for amplification products for HDC (A) and GAPDH (B) obtained from 0.2 μg of total RNA samples from HMC-1 mast (bold line), KU812F basophilic (dot-dashed line), MDA-MB231 breast cancer (dashed line) and HT-1080 fibrosarcoma (thin line) cell lines.

both the HMC-1 and KU812F cells, but there was no detectable amplification of HDC in samples from the HT-1080 and MDA-MB231 tumor cells (Fig. 4A). However, as expected, there was a specific amplification of GAPDH in samples from all the test cell lines (Fig. 4B). It should be underscored, however, that the melting point of the GAPDH amplification product obtained for samples from MDA-MB231 cells was slightly higher (Tm=84.0±0.2˚C) than that obtained for samples from the other three cell lines. The quantification of HDC signals normalized using the GAPDH signal as an internal standard was carried out according to the method described by Liu and Saint (21). These ratio values were 5.30×10^{-3} for HMC-1 and 5.58×10^{-3} for KU812F cells.

Discussion

It is well established that HDC is expressed by mast cells and, therefore, mastocytosis show increased levels of the enzyme (10). Severe mastocytosis and mastocytomas are considered to be oncologic diseases (11). Previously, histidine decarboxylase has been described as a new marker for several leukemia and highly malignant forms of cancer, such as melanoma and small cell lung carcinoma (4-7). All these data underscore the importance of a reliable and automatizable quantitative method to determine HDC expression. However, up until now no such method was available. In this report, we describe a rapid, sensitive, specific, and quantitative assay to determine HDC mRNA levels in biological samples useful for determinations with potential diagnostic value in oncology.

Acknowledgments

This work was supported by funds from Red Española de Mastocitosis (REMA, Spanish Ministry of Health), and research grants SAF2002-02586 from DGICYT (Spanish Ministry of Education and Science) and from Fundación Areces. The iQ cycler instrument was made available by the Lipids and Atherosclerosis research group (CIMES, University of Málaga). Thanks are due to Mrs. María José Ariza for her technical support.

References

1. Medina MA, Urdiales JL, Rodriguez-Caso C, Ramirez FJ and Sanchez-Jimenez F: Biogenic amines and polyamines: similar biochemistry for different physiological missions and biomedical applications. Crit Rev Biochem Mol Biol 38: 23-59, 2003.
2. Ghosh AK, Hirasawa N, Ohtsu H, Watanabe T and Ohuchi K: Defective angiogenesis in the inflammatory granulation tissue in histidine decarboxylase-deficient mice but not in mast cell-deficient mice. J Exp Med 195: 973-982, 2002.
3. Nathan C: Points of control in inflammation. Nature 420: 846-852, 2002.
4. Haak-Frendscho M, Darvas Z, Hegyesi H, *et al*: Histidine decarboxylase expression in human melanoma. J Invest Dermatol 115: 345-352, 2000.
5. Graff L, Frungieri M, Zanner R, Pohlinger A, Prinz C and Gratzl M: Expression of histidine decarboxylase and synthesis of histamine by human small cell lung carcinoma. Am J Pathol 160: 1561-1565, 2002.
6. Tanimoto A, Matsuki Y, Tomita T, Sasaguri T, Shimajiri S and Sasaguri Y: Histidine decarboxylase expression in pancreatic endocrine cells and related tumors. Pathol Int 54: 408-412, 2004.
7. Matsuki Y, Tanimoto A, Hamada T and Sasaguri Y: Histidine decarboxylase expression as a new sensitive and specific marker for small cell lung carcinoma. Mod Pathol 16: 72-78, 2003.
8. Tetlow LC and Woolley DE: Immunolocalisation of histamine and histidine decarboxylase in chondrocytes of arthritic cartilage. Inflamm Res 53 (Suppl 1): S21-S22, 2004.
9. Ohmori E, Fukui T, Imanishi N, Yatsunami K and Ichikawa A: Purification and characterization of l-histidine decarboxylase from mouse mastocytoma P-815 cells. J Biochem (Tokyo) 107: 834-839, 1990.
10. Mamune-Sato R, Yamauchi K, Tanno Y, *et al*: Functional analysis of alternatively spliced transcripts of the human histidine decarboxylase gene and its expression in human tissues and basophilic leukemia cells. Eur J Biochem 209: 533-539, 1992.
11. Valent P, Sperr WR, Schwartz LB and Horny HP: Diagnosis and classification of mast cell proliferative disorders: delineation from immunologic diseases and non-mast cell hematopoietic neoplasms. J Allergy Clin Immunol 114: 3-12, 2004.
12. Tippens AS and Gruetter CA: Detection of histidine decarboxylase mRNA in human vascular smooth muscle and endothelial cells. Inflamm Res 53: 215-216, 2004.
13. Fajardo I, Urdiales JL, Medina MA and Sanchez-Jimenez F: Effects of phorbol ester and dexamethasone treatment on histidine decarboxylase and ornithine decarboxylase in basophilic cells. Biochem Pharmacol 61: 1101-1106, 2001.

ed Biosystems: Relative Quantification of Gene sion. In: User bulletin #2. ABI PRISM 7700 sequence detection system. Applied Biosystems, pp1-36, 2001.
15. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45, 2001.
16. Engels WR: Contributing software to the internet: the Amplify program. Trends Biochem Sci 18: 448-450, 1993.
17. Bustin SA: Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29: 23-39, 2002.
18. Kuramasu A, Saito H, Suzuki S, Watanabe T and Ohtsu H: Mast cell-/basophil-specific transcriptional regulation of human L-histidine decarboxylase gene by CpG methylation in the promoter region. J Biol Chem 273: 31607-31614, 1998.
19. Committee TIS: Text on validation of analytical procedures Q2A. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Available on line at: http://www.ich.org/Main.iser?@_ID=519, 1994.
20. Committee TIS: Validation of analytical procedures: methodology Q2B. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Available on line at: http://www.ich.org/Main.iser?@_ID=519, 1996.
21. Liu W and Saint DA: A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal Biochem 302: 52-59, 2002.