

Short Communication

Expression of Histidine Decarboxylase and Synthesis of Histamine by Human Small Cell Lung Carcinoma

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We recently found that human small cell lung carcinomas (SCLCs) express, in addition to other neuroendocrine markers, vesicular monoamine transporters. Our present results indicate that SCLCs are histaminergic. We detected the biosynthetic enzyme histidine decarboxylase by immunohistochemistry in paraffin sections of 12 biopsies of SCLC tumors. This finding was supported by immunoblotting and reverse transcription-polymerase chain reaction experiments using established SCLC cell lines, frozen and paraffin-embedded SCLC tumors. Moreover, we found histamine to be synthesized, stored, and released by cultured SCLC cells. Our novel observations may be useful for developing new diagnostic tools for this frequent and highly malignant tumor. (Am J Pathol 2002, 160:1561–1565)

Catecholamines, serotonin, and histamine, synthesized in the cytoplasm of aminergic cells, are taken up into intracellular storage vesicles by proton-driven vesicular monoamine transporters.¹ We recently detected both types of known vesicular monoamine transporters (VMAT1 and VMAT2) in human small cell carcinomas (SCLCs), whereas non-SCLC tumors such as large cell carcinomas, adenocarcinomas, and squamous cell carcinomas did not express VMATs.² In the present study we attempted to identify the nature of the amine stored by the high-grade malignant SCLCs.³ The enzymes involved in the biosynthesis of biogenic amines which are synthesized from amino acid precursors are well known. Tyrosine hydroxylase (TH) is the key enzyme in the synthesis of catecholamines, tryptophan hydroxylase (TPH) in that of serotonin, and histidine decarboxylase (HDC) in

that of histamine. Thus, we first determined whether the biosynthetic enzymes are present in SCLCs. Subsequently, we examined the amine suggested by the enzymatic makeup by established human SCLC cell lines. Our results show that histamine is a major secretory product of human SCLCs.

Materials and Methods

Immunohistochemistry and Immunoblotting

Twelve bronchoscopic biopsies of human SCLC tumors, a snap-frozen tumor, and two established SCLC cell lines (see below) shown previously to express SNAREs (SNAP receptors) and VMATs were investigated. Immunohistochemistry and immunoblotting were conducted as described.² The following primary antibodies were used for immunohistochemistry: polyclonal anti-HDC (1:10000, Euro-Diagnostica AB, Malmö, Sweden), monoclonal anti-TH (1:40, Loxo GmbH, Dossenheim, Germany), monoclonal anti-TPH (1:1000, Sigma-Aldrich, Deisenhofen, Germany) and monoclonal anti-tryptase (1:100; DAKO, Hamburg, Germany). For control purposes the first antibody was replaced by nonimmune mouse or rabbit serum in concentrations matching the immunoglobulin concentrations of the specific antibodies used. One such control is shown in Figure 1 G. Paraffin sections of human adrenals, duodenum, stomach, and testis served as positive controls. For immunoblotting, the anti-HDC antibody was diluted 1:8000.

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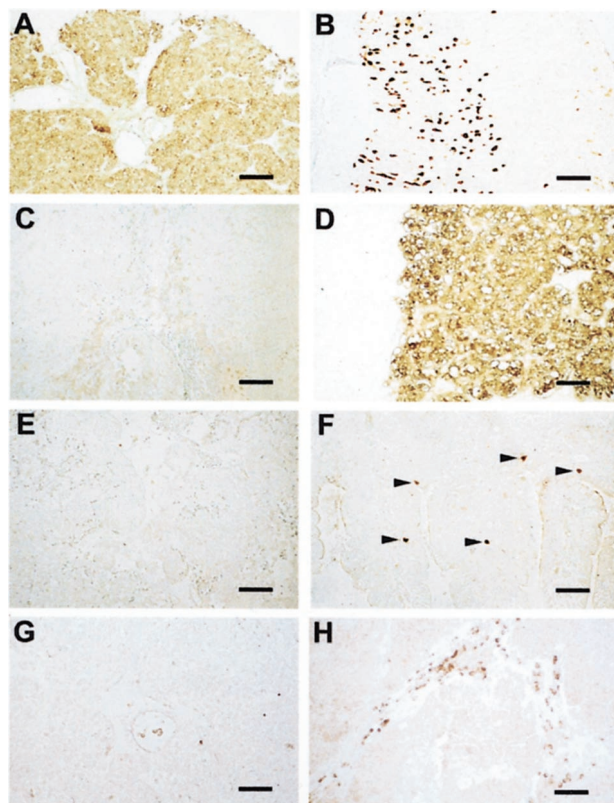


Figure 1. Presence of HDC and absence of TH and TPH in SCLC tumors. Immunohistochemistry of a SCLC tumor (case 11 in Table 1), stomach, adrenal, and duodenum is shown. HDC immunostaining was found in the tumor (A) and in the ECL cells of the human stomach mucosa (B). No staining was observed in the SCLC tumor using antibodies directed against TH (C) and TPH (E), but strong immunostaining is apparent in adrenal medullary chromaffin cells (D) and in enterochromaffin cells (arrowheads) of the duodenum mucosa (F). Nonimmune rabbit serum (1:10,000) is shown as a negative control (G). The location of mast cells is shown by immunostaining with an antibody directed against tryptase. Groups of mast cells present in the stroma of the tumor are shown between the clusters of unstained SCLC cells (H). The size of the bars (equivalent to 100 μ m) is given on the right.

Reverse Transcription-Polymerase Chain Reaction Analysis

RNA from cultured SCLC cell lines (see below) was prepared using the RNeasy kit (Qiagen, Hilden, Germany). RNA (500 ng) from cultured cells was used for reverse transcription as described previously.² The following primers designed to span exons 4 and 5 of HDC (GenBank accession no. M60445) were used: for the first PCR 5'-GAA CGA ATC ATC ATG CCT and 3'-TTC CAC AGA GGA GTG AGC and the primers for nested PCR were 5'-CTA CTA CCC AGC CCT CAC C and 3'-AGG CAG GAC TCA TCA GCA. PCR conditions were as follows: 2 minutes of initial denaturation at 94°C and 34 cycles of 30 seconds, annealing at 56°C, with a 1-minute extension at 72°C. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of paraffin-embedded SCLC tumors was performed as previously described.⁴ In brief, deparaffinized 5- μ m sections of SCLC tumors were scratched from the slides, and RNA was extracted using the Pure-script kit (Biozym, Hessisch Oldenburg, Germany) followed by PCR amplification with the primers described

above. PCR products were subcloned into the pGEMT vector (Promega, Mannheim, Germany) and sequenced using a fluorescence-based dideoxy sequencing reaction (ABI model 377 DNA sequencer; Perkin Elmer, Ueberlingen, Germany).

Histamine Production by SCLC Cell Lines

The human SCLC cell lines SCLC-24H⁵ and NCI-H69⁶, for simplicity termed H24 and H69 in this contribution, were cultivated at a density of 1×10^6 cells/ml in 100-ml flasks (NUNC GmbH, Wiesbaden, Germany) in 4 ml of RPMI-1640 (Sigma-Aldrich, Deisenhofen, Germany) supplemented with 5% fetal calf serum (FCS) or without serum in F12-Dulbecco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 0.5 mg/ml bovine serum albumin, 15 mM HEPES, 50 μ mol/L ethanolamin, 10 μ g/ml insulin, 10 μ g/ml transferrin, and 10 μ g/ml selenit (Sigma) as described.^{5,7} Additional L-histidine (100 μ mol/L; 250 μ mol/L; 500 μ mol/L) was added to the medium to study the influence of the substrate concentration on histamine synthesis. Supernatants and cells were separated by centrifugation at 8000 rpm for 4 minutes. Cells were washed twice with NPE (150 mmol/L NaCl, 10 mmol/L PIPES, 1 mmol/L EDTA) (pH 7.4) and sonified. The samples were stored at -20°C in the dark until measurement of histamine using a commercial radioimmunoassay kit (Immunotech, Marseille, France) with a detection limit of 0.2 nmol/L. The within-assay variability was less than 1%, the between-assay variability less than 5%.

Results

Identification of HDC in Human SCLC

To examine the presence of the key enzymes of amine biosynthesis we performed immunohistochemistry on biopsies ($n = 12$) of SCLCs shown previously to exhibit specific immunoreactivity for VMAT1 and VMAT2 as well as SNAP-25 and syntaxin1.² While the presence of VMATs indicates the possibility of amine storage by the tumor cells, the expression of the SNAREs, SNAP-25 and syntaxin1, is in accordance with the known neuroendocrine nature of SCLCs. Immunostainings of paraffin sections with antibodies directed against HDC, TH, and TPH are shown in Figure 1. In all SCLC tumors HDC was detected (Figure 1A and Table 1). As a positive control we show the presence of HDC in histaminergic enterochromaffin-like (ECL) cells (Figure 1B) of the human stomach.⁸ In contrast, TH and TPH were absent in the tumor tissue (Figure 1, C and E), although both enzymes were readily detected in tissues as positive controls, ie, in human adrenal medullary cells (Figure 1D) and enterochromaffin cells of the human duodenum (Figure 1F). HDC is known to be present also in human mast cells which are, however, distinct from tumor cells. Thus, mast cell location within the tumor tissue is clearly shown by immunostaining with an antibody directed against the mast cell protein tryptase.⁹ In Figure 1H groups of mast

Table 1. Immunohistological Analysis of Paraffin Sections

Section	No.	Age/Sex	HDC	TH	TPH
SCLC biopsies					
	1	56/m	+	-	-
	2	75/f	+	-	-
	3	79/f	+	-	-
	4	95/m	+	-	-
	5	67/m	+	-	-
	6	51/m	+	-	-
	7	64/m	+	-	-
	8	49/f	+	-	-
	9	43/m	++	-	-
	10	70/m	+	-	-
	11	51/m	++	-	-
	12	55/f	+	-	-
Adrenals					
	13	43/m	-	++	-
Stomach					
	14	75/f	-	++	-
Duodenum					
	15		++	-	-
	16		-	-	++

++, strong immunostaining; +, moderate immunostaining; -, no staining.
HDC, histidine decarboxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase.

cells present in the stroma of an SCLC tumor are shown between the clusters of unstained SCLC cells. Our immunohistochemical data are supported by Western blot analysis of SCLC cell lines and a snap-frozen SCLC tumor. We detected two immunoreactive bands of 54-kd and 74-kd in SCLC cell lines H24 and H69 as well as in the SCLC tumor (Figure 2A). Thus, both functional HDC

types with different subcellular localization¹⁰ are present in both samples. Thus HDC in the cell lines can be clearly assigned to SCLC cells, but it cannot be ruled out that mast cells present in the stoma (see above) may have contributed to HDC-immunoreactive bands in tumor biopsies.

To extend the results obtained at the protein level we examined the presence of mRNA coding for HDC by RT-PCR in the SCLC cell lines (H24 and H69) (Figure 2B). Using the primers outlined above, we obtained bands of 305-bp indicating the presence of HDC RNA in the tumor cells. The PCR products were identical with the published data (GenBank accession no. M60445) as determined by sequencing. Moreover, RT-PCR analysis and sequencing with RNA extracted from paraffin-embedded sections of SCLC tumors (Figure 2C), indicated HDC gene expression in SCLC tumors as well as in the mucosa of the stomach which served as a control. As mentioned for the HDC Western blot, in tissue samples, a contribution by tissue mast cells cannot be ruled out.

We conclude from our data that SCLC tumors like the established SCLC cell lines H24 and H69 express HDC. This finding suggests biosynthesis of histamine which can be stored via vesicular monoamine transporters in the storage organelles within the tumor cells.²

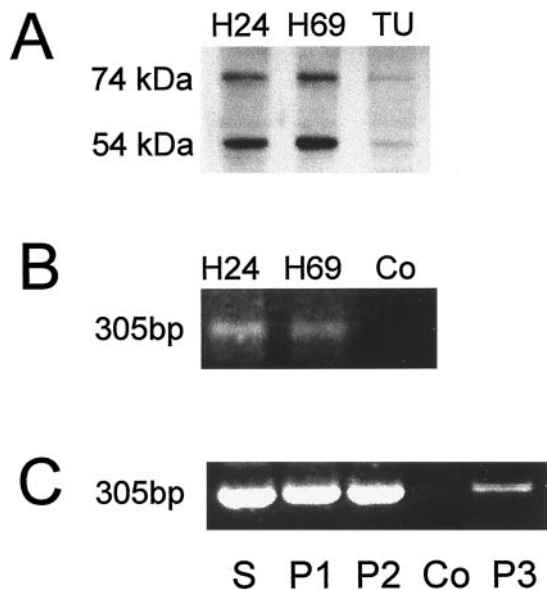
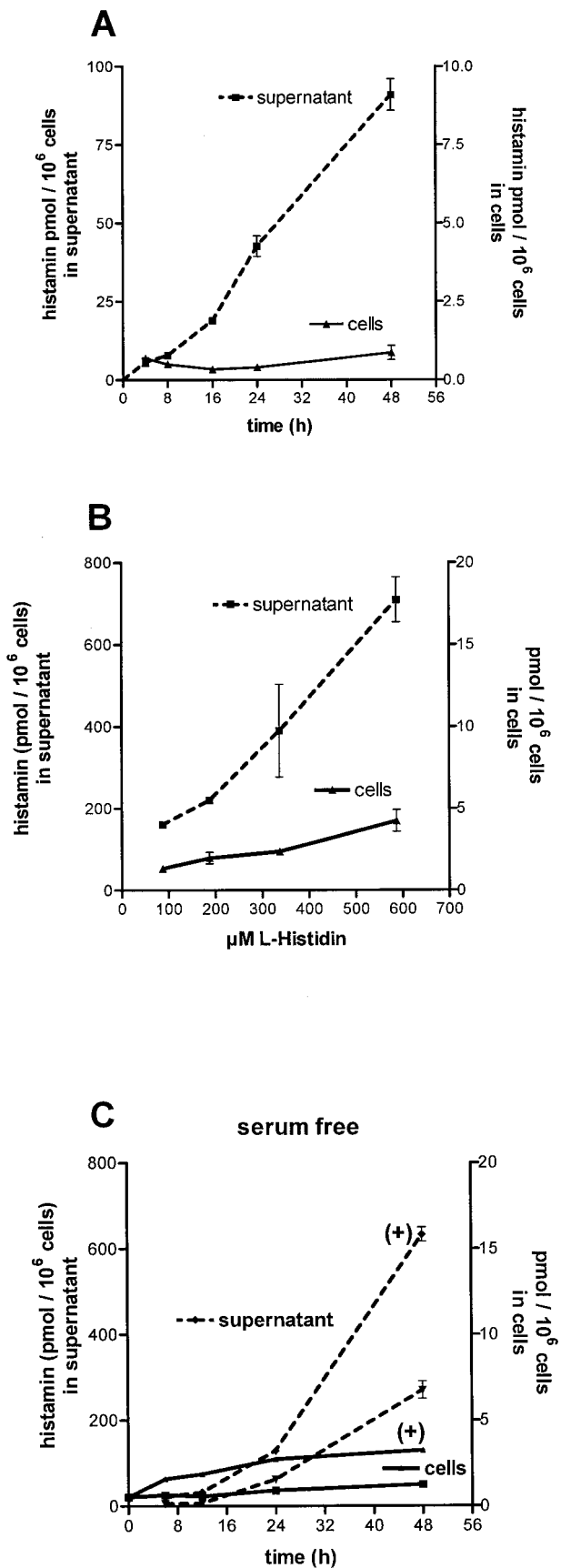


Figure 2. Identification of HDC in SCLC cell lines and SCLC tumors by immunoblotting and RT-PCR. A Western blot of protein extracts of the SCLC cell lines H24 and H69 and a snap-frozen tumor (TU, case 13 in Table 2 of reference 2 is shown in **A**). We observed two bands with the expected molecular weight indicating the presence of both functional forms of HDC. The molecular weight of the bands is indicated on the **left**. We performed nested RT-PCR with mRNA extracted from the SCLC cell lines H24 and H69 (**B**) and of paraffin sections of resected SCLC tumors (**C**) (P1 and P2, cases 2 and 12 in Table 2 of reference 2) and of a biopsy (P3, case 9 in Table 1). We obtained a band of the expected size indicating the presence of RNA coding for HDC. PCR reaction without input cDNA served as negative controls (Co) and stomach (S) as a positive control. The identity of the PCR products was confirmed by sequencing.

Biosynthesis, Storage, and Release of Histamine by SCLC Cell Lines

Direct evidence for the biosynthesis of histamine by SCLC cells was obtained by determination of the histamine content in the cells and in the cellular supernatant with time in culture. In RPMI-1640 medium containing 5% FCS (see Materials and Methods), histamine was continuously released from the cells into the supernatants (Figure 3A) while the amount of histamine within the cells remained roughly constant. However, increasing the lev-



els of L-histidine in the medium, led to an enhanced storage and release of histamine by the cells (Figure 3B). We also determined histamine storage and release in cells cultivated in the absence of serum. In this way, possible effects of factors present in FCS and of serotonin (about 300 nmol/L, released from thrombocytes during the production of the serum) on histamine production can be excluded. Also, in the absence of serum, storage and release of histamine was substantial and of a similar order of magnitude when compared with the values observed in the presence of serum (compare Figure 3A to Figure 3C). Moreover, addition of 500 μmol/L L-histidine caused an increase of histamine in the cells and in the supernatants with and without serum present (compare Figure 3B to Figure 3C). These observations indicate that synthesis, storage, or release of histamine by SCLC cells are not greatly affected by factors present in FCS and serotonin.

Discussion

Neuroepithelial bodies are airway oxygen sensors in the lung, which, like their vascular counterparts in the carotid body, release neurotransmitter during hypoxia. SCLC cells share with sensor cells of neuroepithelial bodies some neuroendocrine properties¹¹ and oxygen-sensitive potassium channels.¹² Neuroepithelial bodies are commonly identified by positive immunostaining for serotonin.¹³ Serotonin was also believed to be a major secretory product of SCLC cells but it is unclear whether serotonin is taken up or synthesized by the tumor cells. Serotonin biosynthesis requires two enzymes, TPH and aromatic amino acid decarboxylase. While the decarboxylase is frequently observed in SCLC tumors,^{11,14} the presence of TPH mRNA¹⁵ but not of TPH protein and its enzymatic activity has been documented in SCLC. Production of histamine by SCLC cell lines as well as presence of HDC in SCLC cell lines and SCLC tumors shown in this study is a novel observation with important implications.

First, the properties of SCLC elucidated in this study may allow definition of the origin of this tumor. Other than mast cells, which are not neuroendocrine in nature, no other histamine producing cell types have yet been detected in the lung. At present it is unclear whether SCLCs are derived from a neuroendocrine precursor cell or from an undifferentiated epithelial cell. Studies on the expression of HDC and of neuroendocrine markers^{2,7,16,17} may be useful in addressing this question.

Second, new markers may be suitable to distinguish SCLC tumors from other lung tumors and to identify primary tumors and metastases of SCLCs in patients. The

Figure 3. Histamine production by SCLC cell lines. **A:** Histamine release by SCLC cells (H69) with time in culture in RPMI-1640 containing 97 μmol/L histidine. **B:** Cell contents and histamine in supernatants after 48 hours in culture were enhanced by increasing the substrate concentration. **C:** Histamine in cells and supernatants was also determined in cells cultivated without serum. Experiments in F12-DMEM medium containing 194 μmol/L histidine and after further addition of 500 μmol/L histidine (+), are shown. Under both conditions histamine in cells and supernatants increased. Values are means of four experiments ± SD.

SNAREs, syntaxin 1 and SNAP-25, and the vesicular monoamine transporters, VMAT1 and VMAT2,² add to other proteins such as synaptophysin, NCAM, chromogranins, and neuron-specific enolase^{7,16,17} which are often variably or inconsistently expressed when investigated in paraffin sections of SCLC tumors. Thus, the World Health Organization defines the diagnosis of SCLC by light microscopic criteria¹⁸. However, newly established markers have been used in related neuroendocrine tumors such as ECL tumors which can be readily identified with a VMAT2 antibody.^{19,20} It is interesting to note that very recently another highly malignant tumor type, human melanoma, has been observed to express HDC.²¹

Substrates of cellular enzymes may serve as tools to examine tumors noninvasively by PET. For example, carcinoids take up ¹¹C-labeled 5-hydroxytryptophan, which allows localization of these tumors in patients.²² Our findings on the presence of HDC (this study) and VMATs² in human SCLC indicate that histidine/histamine-derived tracers may well be used in similar attempts to follow and characterize SCLC tumors and occult metastases in patients.

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