CD97: A Deducification Marker in Human Thyroid Carcinomas

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ABSTRACT

CD97 is a dimeric glycoprotein of Mr 75,000–85,000 and 28,000 belonging to a novel subfamily of seven-span transmembrane region leukocyte cell surface molecules. It is expressed abundantly in cells of hematoepoietic origin. This is the first report demonstrating the expression of CD97 outside the hematopoetic system. CD97 was studied in normal human and neoplastic follicular epithelium of the thyroid and anaplastic (n = 3) and papillary (n = 1) thyroid carcinoma cell lines. In normal thyroid tissue (n = 11), no immunoreactivity of CD97 could be found, whereas in differentiated thyroid carcinomas (n = 10), CD97 expression was either lacking or low. Eleven of 12 undifferentiated anaplastic carcinomas revealed high CD97 presentation. CD97 was absent or only weakly present in patients with postoperative T1 tumors but increased greatly with the progression to postoperative T2 tumors. CD97 is clearly present in thyroid carcinoma cell lines but only at a very low level in normal human thyrocytes. Quantitation of CD97 cell surface expression levels revealed that C 643 and SW 1736 cells showed a two to four times higher specific antibody-binding capacity than did 8505 C and HTB 74 cells and a nearly 20 times higher specific antibody-binding capacity than normal thyrocytes. Phorbol 12-myristate 13-acetate treatment progressively caused a decrease of CD97 antigen expression in all cell lines to about 30% of their initial levels after 48 h. Immunohistochimical staining of SW 1736 cells revealed that CD97 is located in most of the cell compartments and suggested a CD97 internalization process after phorbol 12-myristate 13-acetate treatment. Semiquantitative reverse transcription-PCR showed a correlation of CD97 mRNA and cell surface CD97 expression level in the cell lines. SW 1736, HTB 74, and 8505 C cells apparently expressed CD97 with alternative glycosylation compared to peripheral lymphocytes, whereas most of the CD97 antigen presented on thyrocytes and C 643 cells had glycosylation sites resembling those of lymphocytes. The data suggest that CD97 expression may be a sensitive marker of dedifferentiation and of lymph node involvement in human thyroid tumors.

INTRODUCTION

The leukocyte early activation antigen CD97 (1) is a dimeric glycoprotein of Mr 75,000–85,000 and 28,000. CD97 has thus far been described only in cells of hematopoietic origin. Macrophages, monocytes and granulocytes constitutively express the antigen in high density, whereas peripheral blood lymphocytes bear only a low number of this molecule. Activation of lymphocytes by various stimuli causes a rapid increase of CD97 antigen expression within a few hours (1). The recent cDNA cloning of CD97 (2) and of two other cell surface molecules (3, 4) has identified a novel subfamily of leukocyte antigens with an unusual protein structure (5). This novel structure identifies the molecules as natural protein chimeras, comprising components from two superfamilies: the epidermal growth factor family, and members of the secretin receptor superfamily (2, 4, 6) is restricted largely to the seven-span transmembrane region. On the basis of the distinct structural elements, it is possible to propose a dual ligand interaction for CD97: (a) binding to extracellular matrix components or other cell surface molecules via the epidermal growth factor-like domains and (b) a hormone receptor function mediated by the seven-span transmembrane region. CD97 is a molecule that is potentially involved in adhesion and cell-to-cell signaling processes. This is the first study describing the expression, localization, and antigen characteristics of CD97 on cells outside the hematopoietic system.

MATERIALS AND METHODS

Ethical Approval. The studies described in this paper were approved by the local Committees of Medical Ethics, and all patients gave written consent.

Thyroid Tissues. Thyroid tissue was obtained from patients undergoing surgery for clinical indications of thyroid carcinomas (papillary, n = 4; follicular, n = 6; and anaplastic, n = 12). In one patient, we had the opportunity to study the normal thyroid tissue, primary tumor, and lymph node metastasis. Tissues from benign diffuse goiter and normal thyroid tissue were obtained by surgery of a hyperparathyroidism (n = 11), and tissue from patients with Graves' disease served as control. Histological diagnosis and clinical classification (TNM) including tumor stages (T), regional lymph node metastasis (N), and distant metastasis at the time of surgery (M), as well as postoperative histopathological classification (pTNM), according to the T/ N/ M/ pTNM classification of malignant tumors (Union International Contre Cancer), were realized by the Institute of Pathology, Martin-Luther-Universität Halle-Wittenberg.

Cell Lines and Preparation of Thyrocytes. The following human anaplastic thyroid carcinoma cell lines were analyzed for CD97 expression: C 643 (7), SW 1736 (7), and HTB 74 (8). 8505 C, purchased from the German Collection of Microorganisms and Cell Cultures (DSM ACC219), was established from the primary tumor of a thyroid carcinoma that was characterized histologically as an undifferentiated largely papillary adenocarcinoma with some spindle, polygonal, and giant cells (9). The other cell lines derived from aggressive anaplastic carcinoma. The better differentiation status of 8505 C cells compared to the others is indicated by the fact that only 8505 C cells expressed high levels of thyroperoxidase and thyroglobulin mRNA (data not shown). 8505 C was cultured in RPMI 1640, and the other cell lines were cultured in DMEM with 10% FCS.

Thyrocytes were prepared from two patients with benign diffuse goiters and two patients with Graves' disease as described (10). A thyroid cell suspension resulting from mechanical disaggregation followed by enzymatic digestion was incubated for 18 h in complete RPMI 1640. The adherent fraction obtained after incubation of the cell monolayer with PBS (without Ca<sup>2+</sup> /Mg<sup>2+</sup>) for 45 min was cleared of residual fibroblasts by incubating the cells with the fibroblast-specific monoclonal antibody FIBA501 (11) and goat antieus IgG-DYNABEADS M450 (Dynal, Oslo, Norway) according to the manufacturer's protocol.

CD97 Monoclonal Antibodies. Four monoclonal antibodies, BL-Ac/F2, VIM3, VIM3B, and VIM3C facilitated the definition of the early activation antigen CD97, which was clustered during the Fifth International Workshop on Leukocyte Differentiation Antigens (1, 12). The monoclonal antibody BL-Ac/F2 was used throughout the study.

CD97 Immunostaining of Thyroid Tissues. Tissues were snap-frozen in liquid nitrogen. Frozen sections were cut at 7 μm and fixed in ice-cold methanol for 10 min followed by a short rinse in 0.2 M PBS. They were

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2 To whom requests for reprints should be addressed. Phone: 49-341-9713317; Fax: 49-341-9713219.
prepared for immunohistochemical staining using the monoclonal antibody BL-Ac/F2 following incubation sequences as described (13). The immunoreactivity was evaluated semiquantitatively by two independent investigators blinded to the histological results using light microscope (Axioskop; Zeiss, Jena, Germany). Several sections of tumor tissue from each patient were stained at the same time. Tissue from a patient with a dedifferentiated thyroid tumor was used for each staining procedure as a positive control. CD97 immunoreactivity of the tumor tissue was compared to the positive control and the number of positive cells was judged ranging from 0 to +++ (+, <30% positive cells; +++, 30—70% positive cells; and ++++, >70% positive cells).

RNA Extraction, cDNA Synthesis, and RT-PCR. RNA from 5 x 10^5 cultured cells was isolated by a single-step method using RNAzol B (Biotex Laboratories, Inc., Houston, TX) according to the manufacturer’s protocol. The resulting RNA was washed twice using 80% ethanol, dried, and dissolved in diethylpyrocarbonate-treated water. Five μg of total RNA were taken to synthesize cDNA using the First-Strand cDNA synthesis kit of Pharmacia (Uppsala, Sweden) in a reaction of 15 μl.

To correct for variations across different cDNA preparations, all samples were first adjusted to contain equal-input GAPDH cDNA concentrations. Semiquantitative GAPDH RT-PCR was used with a heterologous synthetic competitor fragment (Table 1). The generation of the specific PCR products from the competitor and the cDNA with the GAPDH primers were published in detail by Platzer et al. (14) and in two studies of our group (10, 15).

We then estimated the CD97 cDNA in these adjusted samples. The CD97 primers were selected according to the previously published sequence for the CD97 antigen (2) using the DNAsis program. The primer pair spans six introns to exclude amplification of genomic DNA.

To quantify CD97 mRNA, a rapid one-step method to synthesize an internal homologous competitor standard was introduced (a schematic of Fig. 1. Schematic for the procedure to synthesize the CD97 mRNA standard; thick arrows, primers.

### Table 1 Primers, length of amplified templates, and assay conditions for RT-PCR

<table>
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<th>Direction</th>
<th>Primer</th>
<th>Length of cDNA (bp)</th>
<th>Length of competitor (bp)</th>
<th>Annealing temperature</th>
<th>Number of cycles</th>
</tr>
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<td>566</td>
<td>64</td>
<td>38</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Hybrid</td>
<td></td>
<td>5' - CTATGAGGTGCCGGACAGGTCTTGTCTTGGAGTCTGTTG - 3'</td>
<td>64</td>
<td>64</td>
<td>38</td>
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*The abbreviations used are: RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SABC, specific antibody-binding capacity; AU, arbitrary unit(s); IL, interleukin; PBMC, peripheral blood mononuclear cell; TSH, thyroid-stimulating hormone; FITC-GAM, FITC-labeled goat antimouse immunoglobulin.*

Fig. 2. Examples of immunoreactivity for CD97 (original magnification, X40). A, papillary thyroid carcinoma tissue with weakly to moderately expressed CD97 (±, <30% positively stained cells, or +). B, CD97-positive cells of a follicular thyroid carcinoma (<30% positively stained cells, or +). C, undifferentiated anaplastic thyroid carcinoma with strongly expressed CD97 (>70% positively stained cells, or +++).
CD97 IN THE THYROID

Table 2 Expression of CD97 in thyroid tissue from patients with thyroid carcinomas determined by immunohistochemistry

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age</th>
<th>Histological diagnosis</th>
<th>pT/N/M</th>
<th>CD97 expression</th>
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<td>17</td>
<td>PTC</td>
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<td>++</td>
</tr>
<tr>
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<td>F</td>
<td>40</td>
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<td>1/0/0</td>
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<tr>
<td>3</td>
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<td>1/0/0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>62</td>
<td>PTC</td>
<td>2/0/0</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>61</td>
<td>FTC</td>
<td>1/0/0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>F</td>
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<td>+++</td>
</tr>
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<td>53</td>
<td>ATC</td>
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<td>+++</td>
</tr>
<tr>
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<td>+++</td>
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<tr>
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<td>M</td>
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<td>ATC</td>
<td>3/0/0</td>
<td>+++</td>
</tr>
<tr>
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<td>F</td>
<td>63</td>
<td>ATC</td>
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<td>++</td>
</tr>
<tr>
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</tr>
<tr>
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<td>++</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>47</td>
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<tr>
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<td>ATC</td>
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<td>+++</td>
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<tr>
<td>22</td>
<td>M</td>
<td>61</td>
<td>ATC</td>
<td>4/0/0</td>
<td>+++</td>
</tr>
</tbody>
</table>

*F, female; M, male; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; ATC, undifferentiated anaplastic thyroid carcinoma; ?, no information; 0, no CD97 immunostaining was found; +, <30% positive cells; ++, 30—70% positive cells, ++++, >70% positive cells; T/N/M, postoperative histopathological classification.

Results

Immunostaining of CD97. In all normal thyroid tissues, immunoreactivity of CD97 was not found. In differentiated thyroid carcinomas (papillary and follicular thyroid carcinoma), the CD97 antigen was detected in six and weakly expressed in four tumors (+, Fig. 2 and Table 2). In 11 of 12 undifferentiated anaplastic thyroid carcinomas the immunostaining of CD97 was scored strongly positive (+++, Fig. 2 and Table 2). However, in one undifferentiated tumor, no immunoreactivity was detected. All six of the CD97-negative differentiated tumors were classified as P1, (tumor diameter of ≤1 cm), 2 with weakly positive staining (+) as P2 (tumor diameter of between 1 and 4 cm). The undifferentiated primary tumors are identified as pT3-4 (tumor diameter of >4 cm or cervical tumor invasion). Patients with weakly positive or negative CD97 immunostaining had neither nodal nor distant metastases and were classified as N0/M0 (n = 6). Distant metastases were found only in one patient with strong staining for CD97 (+++; M1), whereas three pT4 patients with

Followed by a 24-h culture period in FCS-free OPTI-MEM. The medium was aspirated and 10 ng/ml PMA in OPTI-MEM was added to the cultures for another 6 h. The cell monolayers were fixed in 100% methanol for 10 min and treated with the monoclonal antibody BL-AcIF2 (CD97) for 30 min. After washing, a FITC-conjugated F(ab')2 fragment of goat-antimouse immunoglobulin (DAKO) was applied for 30 min at room temperature.

Biochemical Characterization of the CD97 Antigen on the Cell Lines. Surface labeling of cells was performed by a modification of a described method using p-biotin-N-hydroxysuccinimide ester (Boehringer Mannheim; Ref. 17). Cell lysates (5 × 10^7 cells/ml) were prepared in 50 mm Tris-HCl, 0.15 m NaCl (pH 8), 2% NP40, 1 mm phenylmethylsulfonyl fluoride, and 1 mm EDTA and used for immunospecific isolation of the CD97 antigen (18). The absorbed antigens were eluted and subjected to analysis by SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose, which was probed with streptavidin/alkaline phosphatase (Boehringer Mannheim), and processed with 0.5 mg/ml nitroblue tetrazolium and 0.25 mg/mI 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.; Ref. 19). The isolated CD97 antigen was deglycosylated by incubation with 0.5 units of endoglycosidase F (Boehringer Mannheim) in 20 mm phosphate buffer, 50 mm EDTA (pH 6.1), 1% NP40, 1 mm phenylmethylsulfonyl fluoride, and 1% 2-mercaptoethanol for 20 h and analyzed by SDS-PAGE.

Statistics. The CD97 antibody-binding capacity levels and CD97 mRNA levels are presented as the mean ± SE of the different cell types. Significant differences between PMA-treated and untreated cells were evaluated by using the Mann-Whitney U test.

RESULTS

Immunostaining of CD97. In all normal thyroid tissues, immunoreactivity of CD97 was not found. In differentiated thyroid carcinomas (papillary and follicular thyroid carcinoma), the CD97 antigen was detected in six and weakly expressed in four tumors (+, Fig. 2 and Table 2). In 11 of 12 undifferentiated anaplastic thyroid carcinomas the immunostaining of CD97 was scored strongly positive (+++, Fig. 2 and Table 2). However, in one undifferentiated tumor, no immunoreactivity was detected. All six of the CD97-negative differentiated tumors were classified as P1 (tumor diameter of ≤1 cm), 2 with weakly positive staining (+) as P2 (tumor diameter of between 1 and 4 cm). The undifferentiated primary tumors are identified as pT3-4 (tumor diameter of >4 cm or cervical tumor invasion). Patients with weakly positive or negative CD97 immunostaining had neither nodal nor distant metastases and were classified as N0/M0 (n = 6). Distant metastases were found only in one patient with strong staining for CD97 (+++; M1), whereas three pT4 patients with
The ability of competitive PCR to accurately measure relative changes in CD97 mRNA was established using unstimulated and phytohemagglutinin (10 μg/ml)-stimulated PBMCs (Fig. 4). Restriction mapping using several restriction enzymes confirmed identity of the CD97 PCR products. A 4-fold increase in CD97 mRNA was observed after 3 h of activation (PBMC uninhibited, 11.0 ± 2.0 AU; PBMC stimulated, 39.6 ± 3.2 AU; mean ± SE). After 24 h, transcription declined slowly. These data confirmed a previously published study analyzing the CD97 gene transcription by Northern Blot experiments (2).

CD97 mRNA was expressed in all thyrocyte and anaplastic thyroid carcinoma cell line preparations. Quantitation of CD97 mRNA (n = 3) revealed a higher CD97 mRNA level in SW 1736 (40.2 ± 3.0 AU) and C 643 cells (16.0 ± 0.8 AU) compared to 8505 C (10.8 ± 3.6 AU), HTh 74 (3.9 ± 0.5 AU) cells, and in vitro cultured human thyrocytes (10.7 ± 5.0 AU). SW 1736 and C 643 cells presented CD97 at a much higher density on the cell surface compared to the other cell lines. Surprisingly, thyrocytes that expressed CD97 near the detection limit in flow cytometry showed higher CD97 mRNA levels than HTh 74 cells. Furthermore, the CD97 mRNA level was quantitated in SW 1736 cells after 3, 6, and 24 h of PMA exposure. No significant changes could be determined (data not shown).

Expression and Modulation of CD97 on Thyroid Carcinoma Cell Lines. All investigated cell lines expressed CD97. Because the number of the CD97 molecules varied between these cell lines, standard beads mimicking cells with defined antigen densities were introduced to determine the CD97 SABC on the cell surface. The study revealed that the cell lines C 643 and SW 1736 had a 2—4 times higher CD97 SABC compared to 8505 C and HTh 74 and a 15—20 times higher SABC than thyrocytes. The time course of CD97 expression was investigated after addition of various agents to the cell

Strong immunostaining had nodal metastases (N₃). In a N₃ patient, from whom normal thyroid tissue, lymph node metastasis, and primary tumor tissue were available, the CD97 immunoreactivity was almost nondetectable in normal thyroid tissue but strongly positive in primary tumor tissue and in nodal metastasis (Fig. 3).

Semiquantitative RT-PCR for CD97 mRNA. All cDNA samples were adjusted to equal GAPDH mRNA input. Amplification efficiencies were comparable for both the target cDNA and the competitor

Fig. 3. Immunoreactivity for CD97 of thyroid tissues and nodal metastasis of a patient (original magnification, ×40). A, normal thyroid tissue; CD97 immunoreactivity was found only in some thyrocytes (evaluated as 0). B, primary tumor tissue of the same patient strongly stained (evaluated as +++). C, lymph node metastasis of the thyroid tumor of the same patient; the intensity of staining was very strong.

Fig. 4. Typical samples of competitive RT-PCR for CD97 mRNA. Serial dilutions of known amounts of the internal CD97 competitor cDNA (1, 0.16 fg; 2, 0.016 fg; and 3, 0.0016 fg) were added to RT-PCR reaction mixtures containing equal aliquots of sample cDNA. The 331-bp (sample cDNA) and 247-bp (CD97 competitor) PCR products were subjected to electrophoresis on a 1.5% agarose gel after amplification. Competition of the CD97 competitor fragment and sample cDNA was determined by measuring the intensity of ethidium luminescence with a charge-coupled device image sensor and analyzing the data. M, 100 bp ladder.
cultures (Figs. 5 and 6). Interestingly, PMA decreased the level of CD97 on all cell lines within 10 min of stimulation. PMA-mediated inhibition varied between the cell lines tested. After 48 h, only 30–50% of the CD97 SABC was measured compared to PMA-untreated 8505 C, C6 643, and HTH 74 cells. SW 1736 cells showed the lowest level of CD97 SABC after 6 h of stimulation, whereas no significant differences between treated and untreated cells could be measured at 48 h. In contrast to PMA, 4α-phorbol 12,13-didecanoate, a phorbol ester inactive on PKC, did not inhibit CD97 expression. Addition of TSH and forskolin had no effect on any cell line (data not shown). IL-1α and lipopolysaccharide, agents that have been shown to be potent stimulators of tumor necrosis factor α and/or granulocyte macrophage colony-stimulating factor secretion in most of the cell lines (10, 20), did not change the CD97 SABC.

Cultured human thyrocytes from patients with Graves’ disease and nontoxic goiter showed a very low CD97 SABC (6345 ± 1750; n = 4), which also could not be enhanced by TSH, forskolin, IL-1α, or lipopolysaccharide.

Immunostaining of SW 1736 Cells. The CD97 antigen was localized in untreated and PMA-stimulated SW 1736 cells that express CD97 in high density by immunohistochemical staining. The CD97 antigen was found on the cell membrane, in the cytoplasm, and on structures around and within the nucleus in untreated cells. Cell extensions that often form long fibers were CD97 monoclonal antibody immunoreactive, whereas fine structures were visible (Fig. 7A). In contrast, a CD68 monoclonal antibody, which stained the cytoplasm and, slightly, the cell membrane of SW 1736 cells, did not show any reactivity with nuclear structures. Similarly, background controls using the second antibody alone or a nonspecific first antibody were negative. Treatment of SW 1736 cells with PMA caused a change in the cell shape and a different CD97 monoclonal antibody staining pattern (Fig. 7B). CD97 reactivity was concentrated on the cell membrane and in the nucleus. Because flow cytometric studies revealed an up-regulated CD97 cell surface expression, the antigen may be concentrated not at but below the outer cell membrane. This staining pattern could confirm the decreased CD97 expression after PMA treatment in flow cytometry and suggests an internalization process of CD97.

Biochemical Characterization of the CD97 Antigen on the Anaplastic Thyroid Cell Lines. Immunoprecipitation studies on normal and TSH-stimulated (data not shown) thyrocytes using the CD97 monoclonal antibody BL-Ac/F2 revealed a Mr, 83,000–85,000 zone (Fig. 8, Lane 2). Similar results were obtained on the cell lines C 634, HTH 74, and 8505 C. From the corresponding cell lysates, glycoproteins of Mr, 82,000, 81,000/70,000, and 76,000, respectively, were precipitated. In normal thyrocytes and C 643 cells, the identity of CD97 was confirmed using endoglycosidase F digestion of N-linked oligosaccharide moieties (Fig. 8, Lanes 3 and 6), which resulted in a shift of apparent molecular weight to about Mr, 60,000 (major band).

DISCUSSION

The results demonstrate for the first time the CD97 expression on cells outside of the hematopoietic system. CD97 is strongly expressed in dedifferentiated human thyroid carcinomas. The expression level seems to be correlated with the TNM stage of the carcinomas, showing overexpression of CD97 in aggressively growing tumors. The strong expression of CD97 on anaplastic carcinoma cells can be a feature of cell dedifferentiation, and, potentially, structural characteristics of CD97 could be responsible for this phenomenon. Thus, the seven-transmembrane region and the three tandemly repeated extracellular epidermal growth factor-like domains classifies CD97 within a novel subfamily of seven-span transmembrane region cell surface molecules. It can be speculated that the establishment of an aberrant signal transduction pathway-mediated seven-span transmembrane region contributes to a selective growth advantage and may play a role in the development of thyroid carcinomas. On the other hand, the extracellular region of CD97 possesses structural similarities to extracellular matrix proteins. Cell-to-cell interactions either between tumors and host inflammatory cells or between the tumor cells themselves could be mediated through CD97, which might be important for cancer progression or regression. Other studies demonstrate the expression of a number of molecules on thyroid carcinoma cell lines, which were generally not found or were expressed at a lower level by thyroid epithelial cells without stimulation (8, 20–24).

The CD97 mRNA level, determined by semiquantitative RT-PCR, correlates with the CD97 SABC in the investigated cell lines. SW 1736 and C643 cells, which expressed the antigen at a very high density, showed a 5–10 times higher CD97 mRNA level than HTH 74 cells. Surprisingly, thyrocytes that express CD97 at much lower levels showed nearly the same and sometimes even higher CD97 mRNA levels than HTH 74 cells. This discrepancy could in part be due to the standardization with GAPDH transcript levels that are overexpressed in differentiated thyroid cell lines (25, 26). Additionally, we could show that a number of differentiated cells with detectable CD97 mRNA levels did not express the antigen on the cell surface, indicating the existence of extracellular or intracellular competitors and/or mechanisms that prevent the expression of the CD97 glycoprotein.

Furthermore, a former study that demonstrated simultaneously CD97 mRNA down-regulation and prolonged CD97 cell surface expression on peripheral blood lymphocytes suggested that posttranscriptional events influence the CD97 cell surface expression (2).

The only agent that influenced the CD97 expression on the cell surface was PMA. In contrast, IL-1α, tumor necrosis factor α, as well as lipopolysaccharide, substances that have been shown to affect a multitude of cellular functions in human thyrocytes and anaplastic carcinoma cell lines (10, 20), had no effect on CD97 expression. The fact that PMA decreases CD97 is in agreement with a study in which we described a delay of the CD97 expression after PMA stimulation of peripheral blood lymphocytes compared to the immediate up-regulation of CD97 after activation by concanavalin A, phytohemagglutinin, or CD3 monoclonal antibody (1). 4α-Phorbol 12,13-didecanoate, a phorbol ester inactive on PKC, did not influence CD97 expression. The results suggest that PMA down-regulates CD97 through the PKC pathway. PMA substitutes for the natural activators, diacylglycerol and arachidonic acid, of nearly all PKC isoforms. In contrast to diacylglycerol, PMA is metabolically stable. The sustained activation of PKC leads to its proteolysis and inactivation. It is difficult to distinguish between PKC activation and PKC down-regulation. In thyroid cells, PMA has been shown to influence growth, differentiation, basal and TSH-induced iodine transport capacity, iodide organification, and peroxide formation (27–29), but it is not clear whether activation or down-regulation of PKC is responsible for these various effects. Phorbol esters have been shown to cause rapid down-regulation of a number of molecules from various cell types (30–36). There is evidence to suggest that the loss of CD4 from peripheral lymphocytes and of phosphodiesterase-activating factor receptors from neutrophils are due to PMA-induced internalization and shedding or cellular redistribution of these receptors (31, 34). Thus, the PMA-induced CD97 loss from the thyroid cell lines is likely to involve internalization of CD97. This assumption is supported by immunofluorescence staining of PMA-treated SW 1736 cells with the monoclonal antibody BL-Ac/F2. In comparison to untreated cells, a
Fig. 5. Time course of CD97 expression on untreated (control) and PMA (10 ng/ml)-treated human thyroid carcinoma 8505 C cells determined by flow cytometry. A, 10 min; B, 1 h; C, 2 h; D, 24 h; E, 48 h after PMA treatment. Dashed line, control; solid line, staining with the monoclonal antibody BL-AcF2 (CD97).
wide hem around the cells was immunoreactive. Simultaneous loss of
CD97 cell surface antigens could be demonstrated by flow cytometry.
Quantitation of CD97 mRNA indicates the the cell surface CD97 loss
was not accompanied by a down-regulation of CD97 mRNA within
the first 24 h. These results suggest that the CD97 antigen is partly
internalized. However, the mechanism by which CD97 disappears
from the cell surface and its exact fate are not clear.

The finding of a broad intracellular localization of CD97 in SW
1736 cells analyzed by immunohistochemical staining with the mono-
clonal antibody BL-AcIF2 was surprising. The localization of the
CD97 seven transmembrane-spanning domain was believed to be
restricted to cell membranes and not detectable in nuclear membrane
structures. In 1995, reports were published on the identification of G
protein-coupled proteins with a seven-transmembrane-spanning do-
main in purified nuclei, with supporting data indicating the likelihood
of G-protein-coupled signaling across the nuclear envelope (37, 38).

Previous biochemical characterization of CD97 α-chain revealed
Mr 78,000, 82,000, and 86,000 bands on PBMCs (1). Digestion of
N-linked oligosaccharides on CD97 of normal thyrocytes, which
resulted mainly in a major Mr ~60,000 band, suggests that these
molecules represent the same polypeptide chain with different N-
linked carbohydrate moieties. However, O-glycosylation cannot be
excluded, at least in C 643 cells, which also possess molecular forms
of CD97 that could not be degraded completely to Mr ~60,000.
Differences in glycosylation may be specific for distinct stages of
cellular activation, differentiation, or malignant transformation. SW
1736, HTh 74, and 8505 C cells apparently express CD97 molecules
that are glycosylated differently in comparison to peripheral blood
lymphocytes, whereas at least the majority of CD97 molecules on
normal thyrocytes and C 643 cells were found to resemble those of
lymphocytes. Although it cannot be completely excluded that at least
a portion of immunoprecipitated CD97 was detected in consequence
of intracellular labeling, and thus does not represent cell surface-
associated CD97, it is more likely that differences in CD97 glycosy-
lation are displayed at the cell surface. The biological significance of
glycosylation differences remains to be clarified.

In summary, the high expression of differentially glycosylated
CD97 on human anaplastic thyroid carcinoma cell lines compared to
human thyrocytes and our data from a limited number of differenti-
ated and undifferentiated thyroid carcinomas have shown that strong
immunostaining to CD97 antibodies seems to be correlated to the
aggressiveness and lymph node involvement of these tumors. Addi-

Fig. 6. Time course of CD97 SABC on PMA (10 ng/ml)-treated and untreated (control) human thyroid carcinoma cell lines (n = 4). The Mann-Whitney U test was used to determine
the statistical significance between control (C) and PMA (■)-treated cells: A, HTh 74; B, SW 1736; C, C 634; D, 8505 C. Data shown are means; bars, SE. *, P < 0.05; **, P < 0.02.
The CD97 SABC was determined using the DAKO QIFIKIT in flow cytometry.
CD97 IN THE THYROID

Fig. 7. Immunofluorescence staining with the monoclonal antibody BL-AcIF2 (CD97) on the thyroid carcinoma cell line SW 1736 to localize the CD97 antigen in vitro. A, in untreated cultures, the cell membrane, including the cell extensions, the cytoplasm, and structures around the nucleus and the nucleus itself, were stained. B, in PMA-treated cells, a wide hem around the cells is immunoreactive (magnification, ×3530; bar, 2.6 μm).

Fig. 8. Immunoprecipitation of the CD97 antigen. Immunoprecipitates were prepared from NP40 lysates of cell surface-biotinylated PBMCs (PMA stimulated, J), normal thyrocytes (2 and 3), SW 1736 (4), C 643 (5 and 6), HTB 74 (7), and 8505 C (8) cells using the CD97 monoclonal antibody BL-AcIF2. Removal of N-linked carbohydrates was performed by incubating the CD97 antigen precipitated from normal thyrocytes (2 and 3) and C 643 cells (6) with endoglycosidase F. The relative mobilities of Mr markers are indicated (bars).

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