The Expression of Carbonic Anhydrase II in Hematological Malignancies

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ABSTRACT

**Purpose:** Carbonic anhydrases (CAs) are key enzymes that regulate acid-base homeostasis in both normal and pathological conditions. Recent studies have shown that they are functionally involved in the growth and invasion of cancer cells. However, there are only a few publications on CAs in hematological malignancies.

**Experimental Design:** Here we investigated the expression of CA isozymes in six malignant hematopoietic cell lines and malignant blast cells of bone marrow samples collected from patients with acute myeloid leukemia, acute lymphoblastic leukemia, or chronic myelomonocytic leukemia.

**Results:** Because three of the malignant hematopoietic cell lines expressed CA II, we also set out to examine its expression in a series of bone marrow samples. Positive reactions were found in 16 of 26 cases (62%) of acute myeloid leukemia, 11 of 15 cases (73%) of acute lymphoblastic leukemia, and 1 of 2 cases (50%) of chronic myelomonocytic leukemia.

**Conclusions:** The results indicate that CA II expression is not restricted to only one cell lineage but may result from a genetic aberration that occurs in both myeloid and lymphatic lineages or in their progenitor cell. Because CA II is expressed in most patients with leukemic blast cells, CA inhibitors may prove to be of value as an adjunct to chemotherapy for such cancers.

INTRODUCTION

The maintenance of appropriate acid-base homeostasis is a prerequisite for normal cell growth, and recent studies suggest that the control of acid-base balance probably plays an important role in tumorigenesis (1, 2). Microelectrode measurements have shown that the extracellular pH in solid tumors is more acidic than that in the adjacent normal tissue (3), whereas the intracellular pH is identical to that in normal tissue or slightly more basic (4). To generate the pH gradient between the extracellular and intracellular compartments, tumor cells express ion transport proteins, including vacuolar H⁺-ATPase, Cl⁻/HCO₃⁻ exchanger, and Na⁺/H⁺ exchanger (5, 6). Many tumors also express CAs, which catalyze the production of H⁺ and HCO₃⁻ ions in the reversible reaction H₂O + CO₂ ⇌ H⁺ + HCO₃⁻ (1). Eleven enzymatically active CA isozymes have been identified in mammals to date, namely, the cytosolic CA I, II, III, and VII; the mitochondrial CA VA and VB; the secretory CA VI; and the membrane-associated CA IV, IX, XII, and XIV (7).

Previous immunohistochemical studies of CA II have indicated that it is expressed in malignant brain tumors (8) and gastric and pancreatic carcinomas (9–11), and recent evidence has indicated that CA IX and XII are also expressed in some tumors and may be functionally related to oncogenesis (9, 12–18). Ivanov et al. (18) recently hypothesized that tumor-associated CA IX and XII may be implicated in acidification of the extracellular milieu surrounding cancer cells, which would create a microenvironment conducive to tumor growth and spreading. Inhibition of the growth of cancer cells by the direct action of CA inhibitors was originally reported by Chegwidden and Spencer (19), who demonstrated that both methazolamide and ethoxzolamide inhibited the growth of histiocytic lymphoma (U-937) cells. More recently, we have shown that acetazolamide markedly inhibited invasion capacity in four renal cancer cell lines (20), an effect attributable to CA II, IX, and XII, which were expressed in these cells.

Although CA expression in several types of malignancy has recently received increasing attention, there are only a few studies on CAs in leukemias (21–24) that include cancers with a high invasion capacity. Leukemia cells can easily spread from bone marrow via circulation to other organs, but various leukemias differ in their ability to form extramedullary tumors i.e.,...
metastases. If CA activity were essential for invasion by other cancer cells, one could analogously predict that active CA(s) could also function in leukemia cells. The present investigation was undertaken to study CA expression in six malignant hematopoietic cell lines. Once we had found that three of the six cell lines analyzed expressed CA II, we went on to study its expression in a series of bone marrow samples from patients with different types of leukemia.

MATERIALS AND METHODS

Cell Lines. The following five cell lines were obtained from the American Type Culture Collection (Manassas, VA): (a) RPMI 8226 (multiple myeloma, B-lymphoid/plasmacytic lineage, plasma blast); (b) U-937 (histiocytic lymphoma, monocytic lineage, monoblast); (c) Jurkat (T-lymphoblast); (d) MOLT-3 (ALL, T-lymphoblast); and (e) HL-60 (acute promyelocytic leukemia, myeloid blast). The OCI-AML-2 cell line represents myeloid blasts from a patient with acute promyelocytic leukemia and has been described previously (25).

Purification of CAs from Cell Lines. Cultured cells were centrifuged at 15,000 × g for 5 min, and the supernatant was subjected to affinity purification. Inhibitor affinity chromatography was performed using CM Bio Gel A coupled to p-aminomethyl benzensulphonamide, as described previously in detail (26).

Antibodies. The polyclonal rabbit antibodies against human CA I, II, and XII had been produced and characterized previously (27, 28). The monoclonal antibody M75 against human CA IX has also been described previously (29).

SDS-PAGE and Western Blotting. Samples (10 μg) obtained from the inhibitor affinity chromatography were analyzed by SDS-PAGE under reducing conditions according to Laemmli (30). Protein standards for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). The electrophoreses were performed in a Novex XCell SureLock unit (Invitrogen Corp./NOVEX, Carlsbad, CA) using Novex NuPAGE 10% Bis-Tris gels. The proteins were transferred electrophoretically from the gel to a nylon membrane (Millipore, Bedford, MA) in a Novex Blot Module. After transblotting, the sample lanes were first incubated with TBST buffer containing 10% cow colostral whey for 30 min and then incubated with the primary antibody diluted 1:2000 in TBST buffer for 60 min. The membranes were washed five times for 5 min with TBST buffer and incubated for 30 min with a peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) diluted 1:3000 in TBST buffer. After washing four times for 5 min in TBST buffer, the polypeptides were visualized with 3,3'-diaminobenzidine tetrahydrochloride. All of the steps were carried out at room temperature.
Bone Marrow Samples and Immunocytochemistry. Human leukemia cells were collected during diagnostic blastic phase (at diagnosis or relapse) from bone marrow samples of 43 patients (26 patients had AML, 15 patients had ALL, and 2 patients had CMML in refractory anemia with excess of blasts/refractory anemia with excess of blasts (in transformation) phase). The survival rates for the patients in the AML, ALL, and CMML categories, respectively, were as follows: (a) 8 of 26 patients (30.8%); (b) 5 of 15 patients (33.3%); and (c) 0 of 2 patients (0%). The range of follow-up was 1.5–8 years. The samples were taken from the patients during routine diagnostic and treatment evaluation at Oulu University Hospital and classified on the basis of their lymphoid or myeloid morphological appearance, according to the French-American-British classification criteria (31–33). The French-American-British subtypes of AML patients were M0 (n = 1), M1 (n = 5), M2 (n = 5), M3 (n = 1), M4 (n = 9), M5 (n = 2), M6 (n = 1), M7 (n = 1), and unknown (n = 1). In addition, the diagnoses were based on the immunophenotype as assessed by flow cytometry using standard lineage immunophenotype markers. The study was approved by the Ethics Committee of Oulu University Hospital and performed in accordance with the guidelines of the Declaration of Helsinki.

Mononuclear cells were isolated from bone marrow samples by Lymphoprep density gradient centrifugation (Nycomed Pharma AS, Oslo, Norway) and washed with FACSFlow electrolyte sheath fluid (Becton Dickinson, Franklin Lakes, NJ). The cells were fixed and permeabilized with a Fix and Perm kit (Caltag Laboratories, Burlingame, CA) according to the manufacturer’s protocol and spread onto microscope slides. They were then immunostained for CA II using a Histostain-Plus kit (Zymed, South San Francisco, CA) according to the instructions provided in the kit insert. After the immunocytochemical staining, the cell nuclei were slightly counterstained with hematoxylin. The extent and intensity of staining were scored by two of the investigators (M. L. and S. Par.). A negative score (0%) was given to a sample that had no evidence of specific immunostaining. The other categories for the extent of staining were 10%, 10–50%, and 50–100%. The intensity of staining was scored on a scale of 0 to 3 as follows: (a) 0, no reaction; (b) 1 (+), weak reaction; (c) 2 (++), moderate reaction; and (d) 3 (+++), strong reaction.

For double immunofluorescence staining, the cells were first fixed, permeabilized, and spread onto microscope slides as described above. They were then rinsed with PBS, pretreated with a solution containing 0.1% BSA and 0.05% saponin in PBS (BSA-PBS-saponin) for 30 min, and incubated with the primary antibodies [rabbit antihuman CA II serum diluted 1:50 in BSA-PBS-saponin and mouse antihuman CD34 antibody (Zymed) diluted 1:10] for 60 min. The cells were rinsed three times for 5 min with BSA-PBS-saponin and incubated for 60 min with the secondary antibodies [FITC-conjugated swine antirabbit IgG and tetramethylrhodamine isothiocyanate-conjugated goat antimouse IgG (Dakopatts)] diluted 1:30 with BSA-PBS-saponin and mouse antihuman CD34 antibody (Zymed) diluted 1:10] for 60 min. The cells were then rinsed with PBS, pretreated with a solution containing 0.1% BSA and 0.05% saponin in PBS (BSA-PBS-saponin) for 30 min, and incubated with the primary antibodies [rabbit antihuman CA II serum diluted 1:50 in BSA-PBS-saponin and mouse antihuman CD34 antibody (Zymed) diluted 1:10] for 60 min. The cells were then rinsed three times for 5 min with BSA-PBS-saponin and incubated for 60 min with the secondary antibodies [FITC-conjugated swine antirabbit IgG and tetramethylrhodamine isothiocyanate-conjugated goat antimouse IgG (Dakopatts)] diluted 1:30 with BSA-PBS-saponin. After washing twice for 5 min in BSA-PBS-saponin buffer and washing once with PBS, the cells were mounted (mounting medium; Inova Diagnostics, San Diego, CA).
CA) and analyzed by confocal laser scanning microscopy (Zeiss, Göttingen, Germany).

RESULTS

CA Expression in Malignant Hematopoietic Cells.
Triton X-100-solubilized proteins from five previously characterized leukemia cell lines and one lymphoma cell line were subjected to affinity purification and analyzed by SDS-PAGE. The RPMI 8226 and OCI-AML-2 cell lines showed 30-kDa polypeptides, corresponding to the reported molecular mass of cytosolic CA I and II (Fig. 1). The positive polypeptides were further characterized using Western blotting, which revealed strong CA II polypeptides in the above-mentioned cell lines and a weak positive signal in HL-60 cells. Other blots using anti-CA I, anti-CA IX, and anti-CA XII antibodies were negative. It is notable that SDS-PAGE pointed to several polypeptides of higher molecular mass in all of the cell lines analyzed, but these bands were devoid of immunoreactivity in the Western blots.

Immunocytochemistry. Because CA II represented the major CA isozyme in the malignant hematopoietic cell lines, we set out to study bone marrow samples from a series of leukemia patients. The intensities and extents of the staining reaction for CA II in the mononuclear bone marrow cells are presented in Fig. 2. Positive reactions for CA II were seen in 62% of the AML samples, 73% of the ALL samples, and 50% of the CMML samples. Both the mean staining intensity and the percentage of positive cells varied markedly in each patient category. The extent or intensity of staining did not correlate with the mortality rate (data not shown). Examples of stained cells in two patients with AML and one patient with ALL are shown in Fig. 3, A–C. The positive signal is confined to the cytoplasm in blast cells. Double immunofluorescence staining using anti-CA II and anti-CD34 antibodies further confirmed that most CA II-positive cells expressed CD34, a marker of immature blast cells (Fig. 4). The control staining using normal rabbit serum instead of the anti-CA II serum showed no specific reaction (Fig. 3, D and E).

DISCUSSION

CAs have recently become a target for intensive research into carcinogenesis and tumor invasion. The isozymes CA IX and XII have been found to be overexpressed in a variety of epithelial neoplasms (13, 28, 34) and functionally implicated in von Hippel-Lindau-mediated carcinogenesis and tumor hypoxia (12, 18). Cytosolic CA I and CA II have also shown abnormal expression in some carcinomas (8, 11, 35). There are only a few studies focused on CAs in malignant hematopoietic cells, however. CA I, the second most abundant nonhemoglobin protein in erythrocytes, has previously been used as a marker of erythroid differentiation in blast cells (22, 23). Two major lines of investigation have been followed previously with regard to the involvement of CA II in hematological malignancies: (a) first, v-ErbA, a mutant thyroid hormone receptor α, has been thought to contribute to erythroblast leukemia through the repression of erythroid-specific target genes such as CA2 (36–41); and (b) secondly, Hillstrom Shapiro et al. (42) and Biskobing et al. (24) have shown that the myelomonocytic leukemia cell line HL-60 expresses CA II, which was further induced by 1,25-dihydroxyvitamin D3 and phorbol myristate acetate. These results suggested that 1,25-dihydroxyvitamin D3 synergistically interacts with protein kinase C-activated pathways to cause a myelomonocytic precursor to express CA II.

For the present purposes, we first selected six malignant hematopoietic cell lines to determine which CA isozymes are expressed in malignant blast cells. One of the cell lines (HL-60) had previously been reported to express CA II (24, 42). In addition, we found two other CA II-positive cell lines: OCI-AML-2 and RPMI 8226. Interestingly, these cell lines represented blast cells of myeloid and lymphoid cell lineages, respectively, suggesting that CA II expression is not cell lineage specific but rather results from a genetic aberration that is independent of cell lineage deviations. The Western blotting experiments showed that none of the cell lines analyzed expressed CA I, IX, or XII. Ivanov et al. (13) have recently shown that the cell lines HL-60 and U-937 do not express CA9 or CA12 mRNA, which is in line with our observation. To our knowl-
edge, the other cell lines that we analyzed have not been tested previously for the expression of CA isozymes.

The presence of CA II in leukemia cells suggests that it may participate in the regulation of pH homeostasis in these cells. Tumor growth is generally known to involve complex interactions between cells and their microenvironment characterized by a low, acidic extracellular pH. Martinez-Zaguilan et al. (43) demonstrated that human melanoma cells cultured at acidic pH were more aggressive than control cells when tested at the same medium pH. They proposed that a change in intracellular pH caused by extracellular acidification results in activation of gelatinase B, which in turn leads to a higher invasion potential in the cancer cells. Tight control over pH homeostasis in tumor cells and in their immediate vicinity is achieved by means of proton titration/extrusion mechanisms that include intracellular CA and membrane-associated factors such as the plasma membrane proton pump, proton channels, Na"/H" exchangers, and monocarboxylate carriers (13). The importance of CAs in these processes has been demonstrated in vitro using the classical CA inhibitors methazolamide, ethoxzolamide, and acetazolamide, which seem to prevent growth or invasion of cancer cells (19, 20). Supuran et al. (44) synthesized novel CA inhibitors, N,N-dialkylthiocarbamylsulfenamido-sulfonamides, which inhibit cell growth in leukemia, melanoma, non-small cell lung, ovarian, colon, central nervous system, renal, prostate, and breast cancer cell lines. Interestingly, Teicher et al. (45) demonstrated that acetazolamide treatment could be beneficial as an adjunct to cancer chemotherapy because it can produce additive tumor growth delays with anticancer drugs in vivo. Analogously, additional studies should address the possibility of CA inhibitors being able to offer a new tool for combating the growth of leukemia cells by targeting pH regulation, which appears to be one of the primary events in tumorigenesis.

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