Choline Kinase Activation Is a Critical Requirement for the Proliferation of Primary Human Mammary Epithelial Cells and Breast Tumor Progression

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ABSTRACT

Breast cancer is still one of the most important tumors among women in industrialized countries. Improvement in both understanding the molecular events associated with the disease and the development of new additional treatments is still an important goal to be achieved. Choline kinase (ChoK) is increased in human mammary tumors with high incidence, and this activation is associated with clinical variable indicators of greater malignancy. Here, we have investigated the role of ChoK in the development of breast cancer and found that ChoK is both necessary and sufficient for growth factor-induced proliferation in primary human mammary epithelial cells and an absolute requirement for the specific mitogenic response to heregulin in breast tumor-derived cells. These results demonstrate that ChoK plays an essential role in both normal human mammary epithelial cell proliferation and breast tumor progression. Furthermore, inhibition of ChoK shows a strong in vivo antitumor activity against human breast cancer xenografts. Thus, ChoK constitutes a novel bona fide molecular target for the treatment of breast cancer patients.

INTRODUCTION

Choline kinase (ChoK), the enzyme responsible for the generation of phosphorylcholine (PCho) from its precursor choline, is the first enzyme in the Kennedy pathway that renders phosphatidylcholine as its final product. Because phosphorylcholine is the most abundant component of the plasma membrane, this pathway has an essential structural function. Recently, ChoK has also been implicated in cell proliferation playing an important role in mitogenic signal transduction pathways (1). In this sense, generation of PCho from ChoK is an essential event in growth factor-induced mitogenesis in NIH 3T3 cells (2–4) and acts as a mitogen or cooperates with mitogens in murine fibroblasts (5, 6). In addition, several oncogenes such as ras, src, or mos induce an increase of both ChoK activity and intracellular levels of PCho (7–10). A role for ChoK in the generation of human tumors has also been suggested by nuclear magnetic resonance techniques because elevated levels of PCho in some human tumor tissues as compared with corresponding normal tissues have been reported (11–13). In keeping with the role of ChoK and PCho in malignant transformation, we have reported recently that ChoK is up-regulated in human mammary carcinomas with a high incidence (14) as well as in lung, colorectal, and prostate tumors (15). The observation of enhanced ChoK activity in consonance with its mitogenic properties has constituted the basis of the design of a new antitumor strategy focused on specifically inhibiting this enzyme. Thus, ChoK inhibitors have been generated with proven antiproliferative activity against oncogene-transformed cells and human tumor cells (16–19).

Breast cancer is still one of the most important tumors among women in industrialized countries (20). As a consequence of the relevance of breast cancer, it has been the target of many clinical trials, resulting in a reduction in mortality among women with this malignancy. However, improvement in both understanding the molecular events associated with the disease and the development of new additional treatments is still an important goal to be achieved. We have previously investigated the relevance of ChoK in human breast cancer and found that this enzyme is increased in human mammary tumors with high incidence (38.5%). Furthermore, this activation is associated with clinical variable indicators of greater malignancy such as high histologic tumor grade and estrogen receptor-negative tumors (14). To further investigate the involvement of ChoK in receptor-mediated signal transduction pathways that ultimately leads to cell proliferation in the breast epithelium, we provide evidence for a role of ChoK in normal human mammary epithelial cell proliferation and breast tumor progression. In addition, we also demonstrate the therapeutic effect of ChoK inhibitors in human breast cancer cells. The potential use of this antitumor strategy based on ChoK inhibition as a novel approach for treatment of breast cancer patients is discussed.

MATERIALS AND METHODS

Cell Cultures and Transfections. All cell lines used in this study were maintained under standard conditions of temperature (37°C), humidity (95%), and carbon dioxide (5%). Human primary mammary epithelial HMEC cells (Clonetics, San Diego, CA) were grown in MEMB medium supplemented with a bullet kit (Clonetics). Epithelial MCF-7, SkBr3, and MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). T47D cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Transfection of HMEC cells with 2 μg of the indicated genes was carried out using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. Statistical analysis was performed by the Tudey test, using InStat Version 2.04 (GraphPad, San Diego, CA). All factors were treated as single categorical variables, and all reported P values are two-sided. Statistical significance was defined as P < 0.05.

Analysis of Phosphorylcholine Production in Cells. Cells were seeded on 6-well plates and grown in the presence of 1 μCi/mL methyl-[14C]choline chloride (50–60 Ci/mmol; Amersham International, Buckinghamshire, United Kingdom). When indicated, serum-starved cells were labeled with 1 μCi/mL methyl-[14C]choline and, when indicated, exposed to epidermal growth factor (EGF; Sigma, St. Louis, MO), insulin (Sigma), and hydrocortisone (Sigma) or heregulin-β (HRG; Upstate Biotechnology, Lake Placid, NY). Cells were rinsed with ice-cold TD buffer [137 mmol/L NaCl, 5 mmol/L KCl, and 20 mmol/L Tris (pH 7.4)] and fixed with 16% ice-cold trichloroacetic acid (TCA). TCA-soluble material containing PCho was washed three times with 4 volumes of diethyl ether, dried under vacuum, and resuspended in water. Samples were resolved in thin layer chromatography plates (Silica Gel 60 A; Whatman, Clifton, NJ), using 0.9% NaCl:methanol:ammonium hydroxide [50:70.5:5 (v/v/v)] as liquid phase. Radioactivity corresponding to PCho was automatically quantified by an electronic radiography system (Instantimag; Packard, Meriden, CT). TCA-insoluble material containing hydrophobic lipids was dis-
solved in 0.25 N sodium hydroxide, and total lipids were resolved by scintillation counting.

**Analysis of Protein Levels by Western Blotting.** Cells were lysed in ice-cold lysis buffer (50 mM Tris (pH 7.4), 0.25% Nonidet P-40, 0.25% SDS, 150 mM NaCl, 15 mM b-glycerophosphate, 10 mM NaF, 10 mg/mL aprotinin, and 1 mM Pefabloc). Nuclei and detergent-insoluble material were removed by centrifugation at 13,000 rpm for 20 minutes at 4°C. Western blot analysis of equal amounts of cell lysates (30 µg) was performed using each corresponding antibody. A polyclonal antibody against human ChoK was obtained as described in ref. 14. Antibodies against Rb and cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin E and anti-cyclin D3 monoclonal antibodies were from Becton Dickinson (PharMingen International). Phospho-Rb (Ser807/Ser811) antibody was from New England Biolabs (Cell Signaling Technology). Anti-ErbB-3 was from Santa Cruz Biotechnology, and anti-pErbB-3 was from Cell Signaling. Anti-ErbB-2 was from Santa Cruz Biotechnology, and anti-pErbB-2 was from Upstate Biotechnology. As loading control, blots were assayed against a-tubulin (Sigma).

**DNA Synthesis by [3H]Thymidine Incorporation.** Cells were seeded on 6-well plates and, when indicated, transfected with the corresponding DNAs as described previously. After that, cells were washed with TD buffer and incubated in serum-starved medium for 2 days. When necessary, cells were stimulated with each mitogenic factor or a combination of factors and incubated in the presence of 1 µCi/mL methyl-[3H]thymidine for the last 6 hours. When indicated, cells were treated with 30 µmol/L of the specific ChoK inhibitor MN58b 2 hours before stimulation. [3H]Thymidine incorporation into DNA replicating cells was determined as described previously (15).

**Immunofluorescence Assays.** Cells were seeded on 6-well plates over glasses of 12 mm in diameter and transfected with the green fluorescence protein (GFP), as control or with an expression vector encoding GFP-ChoK as described above (see Cell Cultures and Transfections). Forty hours after transfection, cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed three times with PBS and 0.2% Triton X-100. Fixed cells were then permeabilized with 0.2 N HCl for 10 minutes at room temperature and, after being washed four times with PBS and 0.2% Triton X-100, treated with 100% methanol precooled at −20°C for 2 minutes. ChoK expression was detected by confocal microscopy (Leica TCS SPII spectral microscope). Proliferation of these cells was determined by monitoring 5-bromo-2′-deoxyuridine (BrdUrd) incorporation into DNA by fluorescence microscopy (BrdUrd labeling and detection kit; Roche Applied Science, Indianapolis, IN). Treatment of cells and proliferation assay were as indicated by the manufacturers.

**Flow Cytometry Analysis.** After transfection, cells were synchronized in G1/G0 by serum starvation for 46 hours. Cells were trypsinized, centrifuged, and diluted in 1 mL of PBS, and 9 mL of 70% ethanol were added while vortexing. After 24 hours at −20°C, cells were washed with PBS and resuspended in 1 mL of PBS buffer containing 2.5 mMol/L C5H12N3O2S, 0.01% Triton X-100, and 20 µg of propidium iodide. FACScan analysis for relative DNA content based on fluorescence was carried out. Cell cycle analysis was then performed by flow cytometry using a commercially available software package (FACScan; Becton Dickinson, San Jose, CA) as described previously (21).

**Cell Proliferation Assay.** Cells were seeded on 24-well plates at a density of 30 × 10^3 cells/well and incubated for 24 hours under standard conditions. Then, cells were treated with different concentrations of the ChoK inhibitor MN58b (17) and maintained for the indicated time. Quantification of the number of cells remaining in each well was carried out by the crystal violet method as described previously (15). Statistical analysis was performed by the Tukey test, using the GraphPad InStat Version 2.04.

**In vivo Antitumor Assays.** Human breast tumor xenografts were established by subcutaneous injection of tumor cells in nu/nu immunosuppressed mice. Mice were kept under standard laboratory conditions according to the guidelines of the Spanish government. Cells were resuspended in Dulbecco’s modified Eagle’s medium just before inoculation (10^6 cells/0.1 mL). When tumors reached a mean volume of 0.1 cm^3, mice were randomized to control and treatment groups (3 mice per group). Treatments with MN58b (and vehicle for control mice) were performed intraperitoneally with a schedule of daily consecutive doses of 5 mg/kg for 5 days, separated by 9 days. Tumors were monitored at least twice a week by measuring the major (D) and minor (d) diameters, and tumor volume was calculated as V = (D × d^2)/2. The drug was well tolerated by the mice, with no significant effects on general appearance or behavior. Toxicity effects were confirmed by using hairy mice, treated under similar conditions of dose and schedule. No effects on fur, general appearance, or behavior were observed. A small reduction in body weight (~10% loss) was observed.

**RESULTS**

ChoK Is Both Necessary and Sufficient for Growth Factor-Induced Proliferation in Primary Human Mammary Epithelial Cells. Growth factors induce the generation of PCho after stimulation of quiescent murine fibroblasts as a consequence of ChoK activation (2, 3, 22). In addition, PCho itself displays mitogenic activity in mice fibroblasts (2). On the other hand, an association between ChoK activation and the carcinogenic process in human breast cancer has been reported recently (14). All these observations have driven us to...
further study the relevance of PCho production in growth factor mitogenic signaling in primary human mammary cells.

Quiescent primary human mammary epithelial HMEC cells were exposed to a mixture of EGF, insulin, and hydrocortisone, which is required for their normal proliferation. After treatment with these growth factors at concentrations known to stimulate cell proliferation, PCho production was induced with a 60% increase over the basal levels (Fig. 1A). Because an increase in growth factor-induced PCho production has been correlated previously with DNA synthesis in murine fibroblasts (2, 3, 22, 23), we analyzed the ability of EGF, insulin, and hydrocortisone to promote DNA synthesis in HMEC cells treated with MN58b, a ChoK inhibitor (17, 18, 19). MN58b fully abrogated proliferation of these cells under optimal growth conditions (Fig. 1B), when PCho production was drastically inhibited by the specific ChoK interference (Fig. 1C). Thus, ChoK plays a role in growth factor-stimulated proliferation of primary human breast epithelial cells. This effect was also observed after treatment with EGF or insulin alone, although the induction of DNA synthesis was less than that obtained with the complete mixture (data not shown).

Because ChoK activation is required for proliferation of primary human epithelial cells, Chok overexpression and the consequent increase in PCho production should display a mitogenic effect in quiescent HMEC cells. To confirm this, HMEC cells were transiently transfected with vector alone (control) or vector carrying human ChoK (Fig. 2A), and analysis of DNA synthesis by [³H]thymidine incorporation was carried out. When human ChoK (hChoK) was overexpressed in HMEC cells, a >2-fold increase in DNA synthesis was observed (Fig. 2B). We next corroborated that ChoK was inducing primary human mammary epithelial cell proliferation by analyzing BrdUrd incorporation into DNA by fluorescence microscopy. Whereas none of the control cells transfected with GFP were positive for DNA synthesis, similar percentages of cells that were efficiently transfected with GFP-hChoK (19%) underwent DNA synthesis stimulation (22%). Finally, cells expressing GFP-hChoK were also positive for DNA synthesis as determined by BrdUrd-specific fluorescence, a further demonstration that stimulation of DNA synthesis was due to transfection of the hChoK (Fig. 2C and D).

The above-mentioned results suggest that ChoK overexpression is indeed sufficient to promote DNA synthesis in normal human mammary epithelial cells. To further support this conclusion, we
performed an analysis of ChoK-mediated regulation of cell cycle progression. Control and ChoK-transfected HMEC cells were synchronized in G₀-G₁ by serum deprivation for 46 hours. Under these conditions, control cells displayed a larger proportion of cells in G₀-G₁ phase than in S phase or G₂-M phase (Fig. 2E). In contrast, ChoK-overexpressing cells showed a significant displacement of the proportion of cells toward S and G₂-M phases (Fig. 2E). These results, summarized and quantitated in Table 1, demonstrate that overexpression of ChoK is sufficient to induce DNA synthesis and mitotic progression in human primary mammary epithelial HMEC cells.

**Effects of ChoK Expression on Cell Cycle-Regulatory Elements.** Progression through the G₁ phase of the cell cycle and entry into S phase in mammalian cells is controlled by a number of processes and proteins, including phosphorylation of the retinoblastoma protein (pRb), regulated expression of cyclins, and regulation of the activity of cyclin-dependent kinases (Cdks; refs. 24, 25). Cell cycle promotion induced by ChoK in HMEC cells was further investigated in cell extracts from vector- and ChoK-transfected HMEC cells by Western blot analysis using antibodies against some of the key proteins involved in cell cycle control. Overexpression of ChoK induced a clear increase in the levels of both phosphorylated pRb (pRb-P) and cyclin D3 (Fig. 3). However, under similar conditions, no effect was observed on other cell cycle-related proteins such as cyclin D1 or Cdk4. Thus, we can conclude that ChoK is an important molecule in primary human mammary epithelial cell proliferation because its overexpression causes an increase in DNA synthesis, promoting G₁→S transition by at least stimulating phosphorylation of pRb and up-regulating cyclin D3 levels.

**ChoK Is Overexpressed in Breast Cancer Cells with a Consequent Increase in PCho Production.** Once it had been established that ChoK is playing a role in the regulation of human breast normal cell growth, and given that ChoK and PCho have been recently involved in both cellular transformation and human cancer (1, 13–15), we further investigated the role of ChoK in the regulation of breast cancer cell proliferation. In this sense, and to confirm that ChoK is in fact dysregulated in breast cancer, we determined the levels of ChoK in some human breast cancer-derived cell lines and compared them with the levels observed in HMEC cells. All breast tumor cell lines analyzed (MCF-7, T47D, MDA-MB-231, and SkBr3) displayed increased levels of ChoK expression (Fig. 4A) and activity (Fig. 4B) under equivalent conditions of exponential proliferation. Furthermore, PCho production was increased in all four breast tumor-derived cell lines analyzed, with an ~10-fold increase in the intracellular level of PCho, with respect to the levels found in HMEC cells (Fig. 4C).

**ChoK Activation Is Required for Heregulin-Mediated Cell Growth in Human Breast Cancer Cells.** HRG is a growth factor that plays an important role in tumor mammary cell growth via erbB receptors (26, 27). It activates multiple signaling pathways, including extracellular signal-regulated kinase, p38 kinase, and phosphatidylinositol 3'-kinase. Furthermore, HRG has been proposed to increase the expression of proinvasive, prometastatic, and antiapoptotic genes in breast cancer cells, leading to invasive tumor growth (26). To investigate the role of ChoK in the regulation of breast cancer cell proliferation, the intracellular levels of PCho were analyzed in HRG-treated MCF-7 breast tumor cells and in primary HMEC cells. When MCF-7 cells were exposed to HRG within a time and concentration at which HRG-induced mitogen-activated protein kinase (MAPK) activation was observed, the intracellular levels of PCho increased substantially, reaching up to 85% over the basal levels of nonstimulated MCF-7 cells (Fig. 5A and B). By contrast, neither MAPK nor ChoK activation was observed in the primary HMEC cells. This effect was observed even though functional erbB-3 receptor could be detected in HMEC cells after HRG treatment (Fig. 5C). Similar results were also observed when functional erbB-2 was analyzed in HMEC cells under similar conditions (data not shown). Finally, whereas treatment of MCF-7 cells with HRG resulted in a substantial increase in cell proliferation as determined by thymidine incorporation, this growth
factor alone was not able to induce HMEC proliferation, correlating with the inability of HRG to activate ChoK or MAPK (Fig. 5D).

To characterize the relevance of ChoK activation in HRG mitogenic signaling in MCF-7 cells, we next analyzed HRG-promoted DNA synthesis in the presence of MN58b. Treatment with MN58b efficiently blocked HRG-induced MCF-7 cell proliferation (Fig. 5E), suggesting that inhibition of ChoK is sufficient to block the heregulin-stimulated mitogenic signal.

**Inhibition of ChoK Is an Efficient Antitumor Strategy for Breast Cancer Cells Both In vitro and In vivo.** To assess the potential use of ChoK inhibitors (16–19) as antitumor drugs for breast cancer patients, we investigated the sensitivity to ChoK inhibitors of cell lines derived from human breast tumors, and we compared them with that observed in HMEC cells. All breast tumor-derived cell lines investigated showed increased sensitivity to the antiproliferative effect of MN58b with respect to that observed for their corresponding normal cells (Table 2). Furthermore, the viability of the tumor-derived cells was affected in a time-dependent manner, and sensitivity to ChoK inhibition increased, reaching up to 10.5-fold higher than that of the corresponding primary cells. These results indicate that MN58b is an efficient antiproliferative drug against cell lines derived from breast tumors.

We next verified whether inhibition of ChoK could constitute a valid approach for treatment of breast cancer patients. To that end, we tested the in vivo antitumor activity of MN58b against human breast tumor xenografts. Tumors were generated by subcutaneous injection of MDA-MB-231 (an estrogen receptor-negative tumor cell line that is highly tumorigenic in the absence of exogenous estrogens) cells. MN58b was administrated intraperitoneally in sterile 0.9% NaCl. Treatment was initiated when tumors reached a volume of 0.1 cm³ and consisted of five daily consecutive doses of 5 mg/kg, separated by 9 days. Control mice received equivalent volumes of vehicle alone, following an identical schedule. Tumors were monitored twice a week. Treatment with MN58b resulted in a strong inhibition of the tumor growth with an 85% reduction (Fig. 6), indicating that MN58b displays an efficient antitumor activity in vivo against human breast cancer xenografts.

**DISCUSSION**

Advances in cancer research for the last two decades have rendered a better understanding of the mechanisms that regulate cellular proliferation and transformation, with consequent progress in rational drug design. As a consequence, a new age in cancer research and treatment is emerging. In this sense, the identification and characterization of a large diversity of proteins involved in signaling pathways responsible for the onset and progression of cancer have provided new targets for the design of new antitumor strategies. These are based on specific inhibition of the activity of those signaling components essential for the carcinogenic process. A clear example of efficient anticancer drug design is imatinib mesylate (Glivec; formerly STI571). This molecule specifically inhibits the platelet-derived growth factor receptor, c-Abl, and c-Kit kinases and shows efficient antitumor activity in patients with chronic myeloid leukemia, which is associated with the activation of the Bcr-Abl kinase, and in gastrointestinal stromal tumors, which is associated with c-Kit overexpression (29–32). Besides this progress, cancer is still one of the major causes of mortality in developed countries, with an overall rate of near 50% deaths within 5 years of diagnosis. Thus, a better knowledge of the molecular alterations involved in the carcinogenic process is still necessary and will allow us to implement more appropriate treatments for each type of cancer.

ChoK and its product, PCho, have been found to play an important role in mitogenic signal transduction pathways in nontumorigenic murine fibroblasts (1–6) and in murine oncogene-driven proliferation (7–10). Furthermore, a role of ChoK in the generation of human tumors has recently been established (11–14). In particular, ChoK is involved in the development of human breast cancer, with a strong association between ChoK activation and clinical variable indicators of greater malignancy (14).

Here, we have established the relevance of ChoK and PCho in growth factor-induced mitogenic signaling of human cells. First, primary human mammary epithelial HMEC cells display an increase in PCho production in response to EGF, insulin, and hydrocortisone. Furthermore, growth factor-induced HMEC proliferation is completely abrogated by MN58b, a specific ChoK inhibitor developed recently by our group (18, 19). Finally, an increase in PCho production due to ChoK overexpression was sufficient to promote DNA synthesis in HMEC epithelial cells. These results indicate that ChoK activation is both required for growth factor-induced cell proliferation and sufficient for cell proliferation of primary human mammary epithelial cells. Additional studies to establish a spacio-temporal relationship of ChoK regulation and cell cycle progression will help to
achieve a more profound understanding of the relevance of ChoK involvement in the regulation of cell proliferation under normal conditions.

We have demonstrated recently (18, 19) that MN58b is highly specific toward ChoK, with no effect on other enzymes involved in signaling pathways and close metabolic cascades. Furthermore, inhibition of ChoK drastically induces apoptosis in oncogene-transformed and tumor-derived cells, whereas primary human cells are reversibly blocked in G0-G1 (19). Finally, preliminary evidence suggests a rationale for the specificity of MN58b inhibition and involves the generation of ceramides as a possible cause for specific induction of apoptosis in tumor-derived cells versus normal cells (19). These results are highly significant because they establish the basis for the rational use of ChoK inhibitors as novel antitumor drugs. As an additional support, we have attempted to generate ChoK-null cells to further investigate the specific differential requirement of ChoK for normal and tumor cells, but both types of cultured cells are not viable in the absence of ChoK activity; whereas normal cells stop growing, tumor cells dye by apoptosis induction. Thus, there is solid ground to define ChoK inhibitors as rather specific cytotoxic drugs for cancer cells.

Analysis of ChoK-mediated regulation of cell cycle progression showed that ChoK overexpression promotes G1→S transition by up-regulating both phosphorylation of pRb and cyclin D3 levels, two

Fig. 5. ChoK activation is required for heregulin-mediated cell growth in human breast cancer cells. A. Treatment with HRG results in an increase in PCho production in MCF-7 cells. Serum-starved HMEC and MCF-7 cells were labeled with methyl-[14C]choline and, when indicated, exposed to 30 ng/mL HRG for 40 min, and PCho generation was determined. Data are the mean ± variation of three independent experiments in duplicate. B, HRG-induced MAPK activation in MCF-7 tumor cells. Serum-starved HMEC and MCF-7 cells were stimulated with 30 ng/mL HRG for the indicated times. MAPK activation was tested by Western blot analysis using a specific phosphorylated MAPK antibody. A representative blot is shown from three different experiments with similar results. C. Western blot analysis of total ErbB3 levels in both HMEC and MCF-7 cells is shown, as well as the levels of phosphorylated ErbB3 in the presence and absence of HRG after 20 minutes of treatment. Graphs represent the relative amounts of total ErbB3 in HMEC versus MCF-7 cells (left) and the relative amounts of phosphorylated ErbB3 after HRG treatment in HMEC cells (right). D, HRG-induced DNA synthesis in MCF-7 cells. Serum-starved HMEC and MCF-7 cells were treated with HRG (30 ng/mL) for 24 hours and incubated in the presence of 1 μCi/mL methyl-[3H]thymidine for the last 6 hours. Then, [3H]thymidine incorporation into DNA replicating cells was determined. Data show a representative experiment of three independent assays, each performed in triplicate. E. ChoK inhibition efficiently blocks HRG-induced MCF-7 proliferation. Serum-starved MCF-7 cells were treated with 30 μmol/L of the specific ChoK inhibitor MN58b when indicated. Two hours later, cells were exposed to HRG (30 ng/mL) for 24 hours, and [3H]thymidine incorporation into DNA replicating cells was determined after a labeling period of 6 hours. Data show a representative experiment of three independent assays, each performed in triplicate.
INHIBITION OF CHOLINE KINASE IN BREAST TUMOR THERAPY

Table 2  Differential sensitivity to ChoK inhibition of human breast tumor-derived cells and normal human mammary epithelial cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ at 48 h (μM)</th>
<th>IC₅₀ at 72 h (μM)</th>
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<tbody>
<tr>
<td>HMEC</td>
<td>35.6 ± 4.8 (1.0)</td>
<td>16.8 ± 5.1 (1.0)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>6.6 ± 3.7 (5.4)</td>
<td>1.6 ± 0.5 (10.5)</td>
</tr>
<tr>
<td>T47D</td>
<td>6.5 ± 0.2 (5)</td>
<td>1.6 ± 0.2 (10.5)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>12.4 ± 1 (2.9)</td>
<td>5.2 ± 0.2 (3.2)</td>
</tr>
<tr>
<td>SkBr3</td>
<td>5.8 ± 2.1 (6.1)</td>
<td>2.3 ± 0.2 (7.3)</td>
</tr>
</tbody>
</table>

NOTE. Cells were grown under standard conditions in the presence of different concentrations of MN58b. At the indicated times, cells were stained and quantified as described in Materials and Methods. Data represent the corresponding IC₅₀ (μmol/L) after addition of MN58b. Numbers in parentheses indicate the fold sensitivity for each cell line compared with HMEC cells. Data represent the mean of four independent experiments, each performed in triplicate. At any time analyzed, differences were significant between HMEC cells and the other four human tumor-derived cell lines (P < 0.001).

key events in cell cycle progression. Cyclin D3 is the partner of Cdk4 for the promotion of G1→M transition and has been found to be elevated in several tumors such as pituitary tumors, lymphomas, and pancreatic tumors (33). These results are in keeping with our own observations that ChoK inhibition interferes with cell cycle progression at the level of pRb phosphorylation (19). These results make a clear, direct connection of ChoK up-regulation and cell cycle progression, through at least Rb and cyclin D3. However, due to the low efficiency of transfections of HMEC cells used in this study, we cannot rule out the possibility of additional links to other critical components of the regulation of the cell cycle.

In addition, our results also provide evidence for a role of ChoK in the malignant transformation of mammary cells. An increase in the levels of the enzyme and the intracellular levels of PCho is a frequent event in human breast cancer-derived cell lines when compared with the levels observed in primary HMEC cells. Furthermore, ChoK is in fact up-regulated in breast tumors because all four tumor cell lines analyzed (MCF7, MDA-MB-231, T47D, and SkBr3) display increased levels of the enzyme and a drastic increase of up to 10-fold in the intracellular PCho production with respect to the primary cells. These results are in keeping with our previous observation that a large percentage (38.5%) of breast tumors show increased ChoK activity associated with both histologic tumor grade and estrogen receptor-negative status (14).

HRG is a growth factor that has been extensively implicated in the growth of mammary tumor cells (26–28). HRG alone is sufficient to sustain proliferation of tumor-derived mammary cells but is unable to do so in primary HMEC cells. We demonstrate here that HRG-induced mitogenic response in tumor cells requires ChoK because it is completely abrogated by the specific ChoK inhibitor MN58b. However, HRG does not induce MAPK phosphorylation or ChoK activation in primary cells, correlating with the inability of HRG to induce DNA synthesis in this system, under conditions in which it is able to induce activation of erbB receptors. By contrast, and in keeping with previous studies that involve HRG in breast tumor progression (4, 26, 27), HRG alone sustains MCF-7 proliferation. Accordingly, treatment of MCF-7 cells with HRG results in MAPK activation and a strong increase in the intracellular levels of PCho. Finally, HRG-promoted DNA synthesis is efficiently blocked by inhibition of ChoK. These results indicate that ChoK activation is required for HRG-mediated cell growth in human breast cancer cells.

A final strong support to the concept that ChoK is a critical event in breast tumor progression is the evidence that MN58b is a potent in vivo inhibitor of growth of human breast carcinoma cells. We achieved an 85% reduction in tumor growth when MDA-MB-231 cell-injected mice were treated with this specific ChoK inhibitor, without significant toxic effects. This cell line is able to induce tumors in immunosuppressed mice without the requirement for exogenous estrogens, a model that mimics the natural situation in human breast tumors.

Breast cancer is still one of the most important tumors among women in developed countries (34). Although the efficacy of treatment and reduction in mortality have improved greatly in the past decades, a better knowledge of the altered signaling pathways associated with the disease and the development of new treatments are still important goals to be achieved. Here we demonstrate that ChoK is a relevant molecule involved in the development of human breast cancer and that ChoK inhibitors display in vivo antitumor activity against xenografts derived from human breast tumors. These results provide evidence that inhibition of ChoK can be a valid novel chemotherapeutic approach for treatment of breast cancer patients.
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