Sensitivity of Human KB Cells Expressing Platelet-derived Endothelial Cell Growth Factor to Pyrimidine Antimetabolites

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ABSTRACT

Thymidine phosphorylase (dThdPase) is an enzyme involved in pyrimidine nucleoside metabolism. However, little is known about its physiological functions. We previously purified dThdPase from human placenta, isolated a complementary DNA clone for this enzyme, and sequenced it. There was complete sequence identity between 120 amino acids of human dThdPase and the sequence of platelet-derived endothelial cell growth factor (PD-ECGF). Human KB epidermal carcinoma cells transfected with platelet-derived endothelial cell growth factor complementary DNA expressed a 55-kDa protein that was detected with anti-dThdPase antibody and the cell lysate had dThdPase activity. The sensitivity of transfected cells to the antimitabolites was compared with that of untransfected KB cells. The sensitivity of the transfected cells to Doxifluridine (5'-deoxy-5-fluorouridine) was higher than that of untransfected KB cells. Transfected cells were also more sensitive to Tegafur than untransfected KB cells. These results demonstrate that dThdPase is involved in the activation of these antinecancer agents. Since many cancer tissues contain high dThdPase activity compared with normal tissues, these transfected and untransfected KB cells are useful for studying the role of dThdPase in the activation of pyrimidine antimitabolites and also in angiogenesis.

INTRODUCTION

dThdPase catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogues to their respective bases and 2-deoxyribose 1-phosphate (1-3). It also catalyzes deoxyribose transfer from one deoxynucleotide to another base, to form a second deoxyribose 1-phosphate (1-3). It also catalyzes deoxyribosyltransfer to DNA (4-6). Although this enzyme is widely expressed from Escherichia coli to humans, its physiological function in mammals remains unclear. We previously demonstrated that carcinomas in the stomach, colon, and ovary contained higher levels of dThdPase than the normal tissues adjacent to the carcinomas (7). Hammerberg et al. (8) have reported that dThdPase activity in psoriatic lesions increased 20-fold relative to the nonlesional skin. This enzyme may play a critical role in providing the thymidine necessary for proliferation of tumor cells and keratinocytes.

We previously purified dThdPase from the placenta, isolated a cDNA clone for dThdPase, and sequenced it. Our data indicated that residues 125-244 of PD-ECGF are identical to the sequence of human dThdPase (9). We showed that transient expression of PD-ECGF cDNA was cotransfected with plasmid pSV2-neo into KB cells by electroporation (10). After selection with Geneticin (0.56 mg/ml), expression of PD-ECGF/dThdPase in each clone was examined by immunoblotting with anti-dThdPase antibody. One positive clone, KPE-3, and one negative clone, KPE-1, were further analyzed.

Preparation of Cell Lysate. KB and transfected cells were homogenized in hypotonic buffer. After centrifugation, supernatants were assayed for the enzyme activity and resolved by electrophoresis (7). The protein levels were determined by the method of Bradford (17).

Assay of dThdPase Activity. The enzyme activity assay was assayed spectrophotometrically as described previously (7). The enzyme specific activity is expressed as the amount of thymine (nmol) formed/mg protein/h. The enzymatic activity increased linearly between 0.1 and 1 mg cell homogenate protein.

Assay of Thymidine Kinase Activity. Thymidine kinase activity was assayed using a modification of the method of Breitman (18). One unit of thymidine kinase is the amount of enzyme necessary to convert 1 nmol of thymidine to TMP/min.

Immunoblotting. Each sample was resolved by 9.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (19). Proteins in the gel were electrophoretically transferred to a sheet of polyvinylidene difluoride membrane, (Immobilon-P transfer membrane; Millipore, Bedford, MA) with Bio-Rad TRANSBLOT SD as described (20). The membrane was incubated with horseradish peroxidase-conjugated anti-dThdPase antibody (10) and developed using the enhanced chemiluminescence Western blotting detection system (Amersham, Amersham, Buckinghamshire, United Kingdom).

Determination of the Growth Rate of KB and Transfected Cells. Cells were incubated in the 24-well plates, at a density of the 20,000 cells/well, cultured, and then harvested at the indicated times. The cell number was assayed using a modification of the method of Breitman as described (21).
RESULTS

Expression of PD-ECGF DNA in KB Cells. We previously showed that PD-ECGF is identical to dThdPase (9, 10). KB cells were transfected with pSV2neo DNA and PD-ECGF cDNA. We selected six clones that were resistant to Geneticin disulfate (0.56 mg/ml) and determined the dThdPase activity of these clones. Four of the six clones had no dThdPase activity. One of the four was named KPE-1. The other two clones had high dThdPase activity. One of them was named KPE-3. Parental KB cells had no endogeneous dThdPase activity (Table 1). Next, we examined the expression of dThdPase in the lysates of KB, KPE-1, and KPE-3 cells by immunoblotting with anti-dThdPase antibody. Fig. 1 shows that KPE-3 expressed a protein that was specifically detected with anti-dThdPase antibody. The molecular mass (55 kDa) of this protein expressed in KPE-3 cells was identical to that of PD-ECGF in COS cells (data not shown). KPE-1 and parent KB cells did not express detectable amount of dThdPase.

Thymidine Kinase Activity. We investigated whether expression of dThdPase affected the endogeneous thymidine kinase activity. The thymidine kinase activities for KB and KPE-3 cells were 6.55 and 4.18 units/mg protein, respectively (Table 1).

Determination of the Growth Rates of KB, KPE-1, and KPE-3 Cells. We investigated whether the expression of dThdPase affects the growth rate of the cells. The doubling times of KB, KPE-1, and KPE-3 cells were 24.7, 22.3, and 25.7 h, respectively. The growth rate of KPE-3 cells was similar to those of KB and KPE-1 cells.

Drug Sensitivity of KB and KPE-3 Cells. dThdPase may be involved in the activation of pyrimidine antimetabolites. The sensitivity of KB and the transfected cells were determined by the MTT assay. (Table 2). The IC_{50} values of 5-FUra for KB and KPE-3 cells were 11.2 and 7.0 μM, respectively. The difference of sensitivity to 5-FUra between KB and KPE-3 cells was not significant. Since both KB and KPE-3 cells had thymidine kinase activity, we investigated the effect of thymidine on the sensitivity to 5-FUra of the two cell lines. The IC_{50} values of 5-FUra with and without 300 μM thymidine in KB cells were determined. Three hundred μM was the maximum concentration of thymidine that was not cytotoxic to KB cells (data not shown). Significant effects of thymidine on the sensitivity to 5-FUra were not seen in both cells. The sensitivity of KPE-3 cells to FdUrd was 2.8 times higher than that of parental KB cells, but the difference in sensitivity was not significant. The antitumor agent 5'-DFUR is a prodrug of 5-FUra and was converted to 5-FUra by dThdPase (12, 14, 22). The IC_{50} values of 5'-DFUR for KB and KPE-3 were 252 and 13.3 μM, respectively. The sensitivity to 5'-DFUR of KPE-3 cells was 19 times higher than that of parental KB cells. The antitumor activity of Tegafur is reportedly manifested after cleavage to 5-FUra (22). The IC_{50} values of Tegafur for KB and KPE-3 were 163 and 69.3 μM, respectively. The difference in sensitivity was not significant. The antitumor agent 5'-DFUR of KPE-3 cells to Carmofur and 5-fluorouridine was not higher than that of KB cells. The IC_{50} values of vincristine for KB and KPE-3 were similar.

DISCUSSION

The level of dThdPase is reportedly increased in many malignant tumors (24). We also found that some human cancers had higher levels of dThdPase than normal tissues adjacent to the carcinomas. A number of studies on dThdPase in cancer have been performed, because this enzyme is thought to activate pyrimidine antimetabolites (11-13). Expression of this enzyme seems to affect the sensitivity of cells to pyrimidine analogues (11). We have purified dThdPase from human placenta (7) and isolated a partial cDNA clone for dThdPase (10). Full length PD-ECGF cDNA was cloned and sequenced (25). We transfected the PD-ECGF cDNA into epidermoid KB carcinoma cells to express dThdPase. One of the transfected cell lines, KPE-3, had high levels of dThdPase activity and expressed 55-kDa protein that was detected with anti-dThdPase antibody. KB cells had no endoge-

Table 1 Thymidine kinase and dThdPase activity in KB, KPE-1, and KPE-3 cell lysates

<table>
<thead>
<tr>
<th>Drug</th>
<th>KB</th>
<th>KPE-1</th>
<th>KPE-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase activity (units/mg protein)</td>
<td>ND</td>
<td>6.6</td>
<td>4.2</td>
</tr>
<tr>
<td>dThdPase activity (nmol thymine/mg protein/min)</td>
<td>0.0</td>
<td>138</td>
<td>180</td>
</tr>
</tbody>
</table>

a One unit of thymidine kinase is the amount of enzyme necessary to convert 1 nmol of thymidine to TMP/min.

b ND, not determined.
neous dThdPase activity. The sensitivity to pyrimidine antimetabolites of KPE-3 cells was compared with that of untransfected KB cells. The sensitivity to 5'-DFUR of KPE-3 cells was 19 times higher than that of the untransfected cells. The conversion of 5'-DFUR to 5-FUra by dThdPase seems to be more effective on activation of 5'-DFUR than to FUDP by pyrimidine kinase and converted to FdUDP by ribonucleotide reductase. Another route of 5-FUra activation to FdUMP is catalyzed by thymidine phosphorylase and thymidine kinase. The relative importance of the two mechanisms, inhibition of thymidylate synthase by FdUMP and incorporation of FUTP into RNA for toxicity of 5-FUra are disputed.

Kufe et al. (26) have demonstrated a significant relationship between the incorporation of 5-FUra into total RNA and the loss of clonogenic survival of the human MCF-7 breast carcinoma cell line. This correlation was maintained in a similar study in the presence of 10 μM thymidine which should prevent any cytotoxic effects from inhibition of thymidylate synthase. Thus, they interpreted that the incorporation of 5-FUra in RNA is the major mechanism of cytotoxic action in this cell line. Akazawa et al. (27) also demonstrated a positive correlation between the extent of incorporation of 5-FUra into RNA and its cytotoxic effect. They demonstrated that the target of 5-FUra for the cytotoxicity was not thymidylate synthase by using a mutant strain of mouse mammary tumor cell line FM3A that is deficient in thymidylate synthase. On the other hand, Spears et al. (28) developed a sensitive assay system using [3H] FdUMP to detect the thymidylate synthase level in tissues. They showed that tumor sensitivity to 5-FUra is apparently related to virtual ablation of this enzyme activity. The relative importance of the above two mechanisms for 5-FUra toxicity may vary among normal and tumor cells.

Thymidine only slightly decreased the toxicity of 5-FUra to both KB and KPE-3 cells. These results suggested that FdUMP is not the predominant cytotoxic metabolite of 5-FUra in both cell lines. We have shown that 5-FUra cells were more sensitive to drugs that are converted to 5-FUra by dThdPase (5'-DFUR and Tagafur) but not to vincristine which is not activated by dThdPase. Our results indicated that thymidine phosphorylase activates 5'-DFUR and Tagafur in KPE-3 cells.

Some cytokines, such as α-interferon, are reported to increase thymidine phosphorylase activity in human carcinoma and normal cells (29). These cytokines may enhance the sensitivity of the human cancer cells to 5'-DFUR and Tagafur. Further study is needed to elucidate the mechanism for the regulation of dThdPase expression in cancer cells.

REFERENCES

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