Orotate phosphoribosyltransferase localizes to the Golgi complex and its expression levels affect the sensitivity to anti-cancer drug 5-fluorouracil

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ABSTRACT

Orotate phosphoribosyltransferase (OPRT) is engaged in *de novo* pyrimidine synthesis. It catalyzes orotidine to uridine monophosphate (UMP), which is used for RNA synthesis. *De novo* pyrimidine synthesis has long been known to play an important role in providing DNA/RNA precursors for rapid proliferative activity of cancer cells. Furthermore, chemotherapeutic drug 5-fluorouracil (5-FU) is taken up into cancer cells and is converted to 5-fluoro-UMP (FUMP) by OPRT or to 5-fluoro-dUMP (FdUMP) through intermediary molecules by thymidine phosphorylase. These 5-FU metabolites are misincorporated into DNA/RNA, thereby producing dysfunction of these information processing. However, it remains unclear how the subcellular localization of OPRT and how its variable expression levels affect the response to 5-FU at the cellular level. In this study, immunocytochemical analysis reveals that OPRT localizes to the Golgi complex. Results also show that not only overexpression but also downregulation of OPRT render cells susceptible to 5-FU exposure, but it has no effect on DNA damaging agent doxorubicin. This study provides clues to elucidate the cellular response to 5-FU chemotherapy in relation to the OPRT expression level.

Orotate phosphoribosyltransferase (OPRT), which is involved in the pyrimidine *de novo* synthesis pathway, catalyzes the formation of orotidine 5'-monophosphate (OMP), which is subsequently converted to uridine 5'-monophosphate (UMP) by OMP decarboxylase (5) (Fig. 1A). In microorganisms, OPRT exists as a monofunctional protein, while all multicellular eukaryotic OPRT is encoded by a bifunctional protein fused to OMP decarboxylase (15). Furthermore, it is noteworthy that in the history of cancer biology, Heidelberger and coworkers reported an insightful observation that rat hepatoma tumors incorporate uracil into DNA to a significantly great-

er extent than corresponding normal tissues (6, 7). In human cancer cells, *de novo* pyrimidine synthesis plays a pivotal role in providing DNA and RNA precursors for maintaining rapid proliferation. Therefore, it was postulated that a chemically modified uracil molecule might be effective for disrupting tumor DNA synthesis and that 5-fluorouracil (5-FU), a member of the fluoropyrimidine class of anti-cancer agents, exerts cytotoxic activity. Actually, inhibitors targeting components within the *de novo* pyrimidine synthesis have validated this pathway because they arrest the growth of rapidly dividing cells by restricting the pyrimidine supply (13). In addition, in-

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Abbreviations:
dTTP, deoxythymidine monophosphate; 5-FU, 5-fluorouracil; UMP, uridine monophosphate; FUMP, 5-fluoro-UMP; FdUMP, 5-fluoro-dUMP; FUTP, 5-fluorouridine triphosphate; OMP, orotidine 5'-monophosphate; OPRT, orotate phosphoribosyltransferase

Fig. 1 *De novo* pyrimidine synthesis (A) and metabolic pathway of 5-FU (B). (A) OPRT catalyzes the formation of OMP and simultaneously converts OMP to UMP. TS catalyzes *de novo* synthesis of dTMP, which is an essential precursor for DNA synthesis. (B) 5-FU is converted to FUMP by OPRT or further to FdUMP through intermediary molecules by TP. FdUMP inhibits TS, resulting in DNA dysfunction. FUMP can be converted to FUDP and FUTP, which is subsequently incorporated into RNA, resulting in RNA dysfunction. Thin arrows represent attenuated metabolic flows. dTMP, deoxythymidine monophosphate; FdUrd, 5-fluoro-2'-deoxyuridine; 5-FU, 5-fluorouracil; UMP, uridine monophosphate; FUMP, 5-fluoro-UMP; FdUMP, 5-fluoro-dUMP; FUDP, 5-fluorouridine diphosphate; FUTP, 5-fluorouridine triphosphate; OMP, orotidine 5'-monophosphate; OPRT, orotate phosphoribosyltransferase; TP, thymidine phosphorylase; TS, thymidylate synthase.

inhibitors acting on this pathway have been applied successfully to the treatment of malignant neoplastic and autoimmune diseases (3). In this sense, 5-FU primarily exerts an anti-tumor action by inhibiting thymidylate synthase, an enzyme that catalyzes *de novo* synthesis of deoxythymidine monophosphate (dTMP) from dUMP because thymidylate synthase is inhibited by folate and nucleotide analogs such as 5-fluorouridine-dUMP (FdUMP), the active metabolite of 5-FU (12).

Further studies revealed that 5-FU metabolites are incorporated into DNA and/or RNA, thereby leading to dysregulation of DNA/RNA synthesis and function (1) (Fig. 1B). 5-FU is taken up by cancer cells and is phosphorylated to 5-fluorouridine-5'-monophosphate (FUMP), which is the first rate-limiting

step to elicit anti-cancer effects of 5-FU. Actually, OPRT plays a key role in this phosphorylation process. Therefore, together with thymidylate, OPRT is an important factor determining the anti-cancer effects of 5-FU (4, 9). Previous reports have described that enhanced OPRT activation is correlated with an increased response to 5-FU in cancer patients and that this enzyme might be a predictive marker for the response of 5-FU. However, fundamental questions related to the subcellular site of action and the cellular response to variable levels of this enzyme at the cellular level remain unanswered.

This study investigated the subcellular localization of OPRT and elucidated how expression levels of this enzyme affect sensitivity to anti-cancer agents. To this end, we generated a specific antibody to human OPRT and conducted immunocytochemical analyses using various organelle markers. Additionally, we upregulated and downregulated OPRT expression levels in HeLa cells using full-length cDNA or RNAi-mediated silencing of OPRT. We examined the sensitivity to 5-FU and DNA damaging agent doxorubicin (DOX), two major anti-tumor agents acting on different pathways.

MATERIALS AND METHODS

Antibody production. The cDNA fragment encoding the mid region of hOPRT (amino acid residues 86–119) was obtained by polymerase chain reaction using hOPRT/pAcGFP1-C1 expression vector. The cDNA fragment flanked by BamHI and EcoRI sites was subcloned into pGEX-4T-2 vector (GE Healthcare, Piscataway, NJ) for expression of glutathione *S*-transferase (GST) fusion protein, and was sequenced to confirm the reading frame. GST fusion protein was expressed and purified using glutathione-Sepharose 4B (GE Healthcare), according to the manufacturer's instructions. GST fusion proteins were emulsified with Freund's complete or incomplete adjuvant (DIFCO, Detroit, MI) and immunized subcutaneously to female Japan White rabbits at intervals of 2 weeks. After the fifth injection, a polyclonal antibody specific to OPRT was affinity-purified using GST-free peptide coupled to CNBr-activated Sepharose 4B (GE Healthcare). GST-free peptides were prepared by in-column thrombin digestion of GST fusion proteins bound to glutathione-Sepharose 4B media.

Cell culture and immunoblot analysis. HeLa, HEK293, and COS7 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose,

10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin sulfate at 37°C and 5% CO₂. After incubating, cells were lysed in lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 1 mM β -glycerophosphate pentahydrate, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich Co., Saint Louis, MO) (10). Protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL) according to the instruction manual. Equal amounts of protein lysate (30 μ g) were applied in SDS-PAGE and electrophoretically transferred on polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking the non-specific binding sites with 4% non-fat dry milk (w/v) in phosphate-buffered saline (PBS) containing 0.02% sodium azide and 0.2% Tween 20, the membrane was incubated for overnight at 4°C with rabbit anti-OPRT (0.5 μ g/mL), rabbit anti-cleaved poly (ADP-ribose) polymerase (PARP) (1 : 1000, #5625; Cell Signaling Technology, Danvers, MA), or mouse anti- β -actin (1 : 5000, A5441; Sigma-Aldrich Co.) in 2% non-fat dry milk (w/v) in PBS containing 0.02% sodium azide and 0.1% Tween 20. Sites of antigen-antibody reaction were visualized using the chemiluminescent Immobilon Western blotting detection system (Millipore).

RNA silencing. Human specific OPRT siRNA (5'-G CUGUUGCAGCUAGCAGAUGCUUUA-3') was obtained from Life Technologies (Carlsbad, CA). Control siRNA (All stars negative control) was purchased from Qiagen (Valencia, CA). The siRNAs were transfected using RNAiMAX (Life Technologies) according to the manufacturer's instructions (10, 16).

Construction of expression vector. To construct hOPRT/pAcGFP1-C1, a mammalian expression vector which encoded N-terminally AcGFP1-tagged full length of human OPRT, the nucleotide sequence encoding human OPRT (Accession No. NM000373) (14) was amplified by PCR with primers (5'-ATG GCGGTCGCTCGTGCAGCTTTGGGGCCA-3' and 5'-TCAAACACCAAGTCTACTCAAATACGCTTC-3') and HeLa cells first-strand cDNA library as a template. The purified PCR product was subcloned into pGEM-T easy (Promega, Madison, WI). The EcoRI-digested fragment of OPRT was subsequently subcloned into the EcoRI site of pAcGFP1-C1 vector (Clontech Laboratories, Inc., Palo Alto, CA). The cDNA for AcGFP1-tagged OPRT (OPRT/pAcGFP1) was transfected in COS7 cells using Lipofectamine

2000 reagent (Life Technologies) (8). After SDS/12% PAGE, cell lysate proteins (30 μ g per lane) were transferred to PVDF membranes and analyzed by immunoblot using anti-OPRT (0.5 μ g/mL) or mouse anti-AcGFP1 (1 : 5000, #632381; Clontech Laboratories, Inc.) antibody.

Immunocytochemistry. HeLa cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min at room temperature, and permeabilized with 0.3% Triton X-100 in PBS for 10 min. Cells were then treated with 5% normal goat serum in PBS to block nonspecific binding sites, and were incubated with rabbit anti-OPRT (1 μ g/mL) and mouse anti-Golgin-97 (1 : 150, A21270; Life Technologies) or mouse anti-protein disulfide isomerase (PDI; 1 : 200, SPA-891; Stressgen, Victoria, Canada) antibodies containing 0.1% Triton X-100 overnight at 4°C in a moist chamber. After washing with PBS three times, cells were incubated with anti-rabbit IgG-Alexa 488 (green, 1 : 200; Life Technologies) and anti-mouse IgG-Alexa 594 (red, 1 : 200; Life Technologies) for 1 h. Cells were also treated with DAPI (Life Technologies) to stain the cell nuclei. Images were taken with a confocal laser scanning microscope (LSM700; Carl Zeiss Inc., Jena, Germany).

RESULTS

Polyclonal antibody was raised in a rabbit against human OPRT in this study. In immunoblot analysis, a single band was detected on lysates from African Green Monkey Kidney COS-7 cells transfected with human OPRT cDNA, but not on cell lysates transfected with pAcGFP1 vector alone (Fig. 2A). In cell lines derived from human origin, such as HeLa and HEK293 cells, it recognized a single protein band at 52 kDa (Fig. 2B), which corresponds to the reported molecular mass of OPRT. To examine the specificity for the anti-OPRT antibody, we performed RNAi silencing with siRNA for OPRT (siOPRT). In cell lysates from HeLa and HEK293 cells transfected with siOPRT, OPRT protein level was downregulated significantly, suggesting the specificity of this antibody (Fig. 2C). However, the antibody detected no band in cell lysates from mouse or rat cell lines (data not shown). Taken together, this antibody reacts specifically with human OPRT, but not with other species examined.

Next, we determined subcellular distribution of OPRT in HeLa cells by confocal microscopy. The OPRT immunoreactivity was localized to the juxta-nuclear position as a granular structure (Fig. 3A, D).

Fig. 2 Specificity of anti-OPRT antibody. (A) AcGFP1-tagged full-length human OPRT (OPRT/pAcGFP1) was transfected in COS-7 cells. Immunoblot analysis using anti-AcGFP1 antibody confirms proper expression of the construct in transfected cells. Anti-OPRT antibody detects a single band in cells transfected with OPRT/pAcGFP1, but not with vector alone. (B) Immunoblot on cell lysates (30 μ g/lane) from HeLa and HEK293 cells using rabbit anti-OPRT antibody. (C) OPRT siRNA (siOPRT) or control siRNA (siCont) was transfected in HeLa and HEK293 cells. In cell lysates from HeLa and HEK293 cells transfected with siOPRT, OPRT protein level is significantly downregulated. The position of standard protein markers is indicated to the left (kDa).

To examine detailed localization further, we performed double immunolabeling using various markers for organelles. Results showed that OPRT immunoreactivity is mostly colocalized with Golgin-97, a marker for the Golgi complex (Fig. 3A–C). The immunoreactivity was not colocalized with protein disulfide isomerase (PDI), a marker for the endoplasmic reticulum (ER) (Fig. 3D–F). In addition,

other markers, such as mitochondria and lysosome, did not show any similarity to OPRT staining (data not shown). These data suggest that OPRT specifically accumulates near the Golgi complex.

Several lines of evidence have shown that OPRT is implicated in the metabolism of anti-cancer drug 5-FU. Recent studies revealed that OPRT is a main enzyme responsible for the phosphorylation of 5-FU and the sensitivity to this drug for the treatment of human cancer cells (4, 9). Therefore, we next investigated functional implication of OPRT and asked how OPRT expression levels affect susceptibility to anti-cancer agents. To this end, we evaluated the effect of overexpression and downregulation of OPRT using two anti-cancer drugs, doxorubicin (DOX, 0.2 μ g/mL) and 5-FU (5 μ M). First, full-length OPRT was transfected into HeLa cells (Fig. 4A). After treatment with DOX for 24 h, OPRT overexpression had no apparent effect on an apoptotic marker PARP fragmentation level compared with the control (Fig. 4A, lane 3 versus lane 4). However, in response to 5-FU treatment, OPRT-overexpressed cells showed an approximately two-fold increase in PARP fragmentation (Fig. 4A, lane 5 versus lane 6). Second, HeLa cells were transfected with siRNA for OPRT (siOPRT) to downregulate OPRT expression level (Fig. 4B). After DOX treatment, apoptotic marker levels were comparable between the OPRT-downregulated cells and the control cells (Fig. 4B, lane 3 versus lane 4). It is noteworthy, however, that after 5-FU treatment OPRT downregulation engender a more than two-fold increase in PARP fragmentation level (Fig. 4B, lane 5 versus lane 6). Together, these results suggest that both upregulated and downregulated levels of OPRT render cells susceptible to cell death specifically in response to 5-FU exposure, but not to DNA damage induced by DOX.

DISCUSSION

5-FU is a widely used anti-cancer agent for the therapy of various malignancies, including gastric cancers, colorectal cancers, mammary cancers, non-small cell lung cancers, and head and neck cancers. The anti-cancer activity of 5-FU is closely associated with the intratumoral expression of 5-FU-metabolizing enzymes, including thymidylate synthase, OPRT, and dihydropyrimidine dehydrogenase (11). Clinical reports have described a relation between the expression or activities of these enzymes in the tumors and the clinical response or survival of cancer patients upon 5-FU-based chemotherapy (4, 9).

In the present study, we show at the cellular level

Fig. 3 Localization of OPRT in HeLa cells. Green fluorescence represents OPRT. Red fluorescence represents Golgi complex marker Golgin-97 or endoplasmic reticulum marker protein disulfide isomerase (PDI) as indicated. OPRT-immunoreactivity is localized to the juxtannuclear position as a granular structure (A, C, D, F). OPRT-immunoreactivity is mostly colocalized with Golgin-97 (A–C), but not with PDI (D–F). Blue fluorescence represents nuclear staining with DAPI. Scale bars, 10 μ m.

that overexpression of OPRT specifically enhances susceptibility to 5-FU, although it has no effect on DNA damaging DOX as evaluated by an apoptotic marker. This finding is consistent with the previous study, which showed that OPRT overexpression increases 5-FU sensitivity in a cell viability (MTT) assay (18). Unexpectedly, it is apparent that knock-down of OPRT also increases cytotoxicity to 5-FU. These results suggest that not only a high expression level but also low expression levels of OPRT render cells susceptible to 5-FU exposure. How can we understand this phenomenon?

5-FU is phosphorylated in cells to become an active metabolite that inhibits DNA synthesis and which induces RNA dysfunction. After entering cells, 5-FU is converted to FdUMP through intermediary molecules by thymidine phosphorylase. FdUMP then forms a complex with thymidylate synthase, leading to inhibition of DNA synthesis. In addition, 5-FU can be phosphorylated mainly by OPRT to form FUMP; then to 5-fluorouridine triphosphate (FUTP), which is subsequently incorporated into RNA, resulting in RNA dysfunction. Therefore, it is conceivable that the high OPRT level facilitates

5-FU phosphorylation and subsequent production of FUMP and FUTP, leading to RNA dysfunction. However, the low OPRT level might shift the 5-FU metabolic pathway toward the production of FdUMP, which inhibits thymidylate synthase, thereby leading to DNA dysfunction. Increased vulnerability of OPRT-downregulated cells to 5-FU can be explained plausibly by enhanced DNA dysfunction.

Considering prolonged survival of cancer patients receiving 5-FU chemotherapy, two factors must be considered: one is responsiveness of cancer cells to this agent; the other is vulnerability of non-cancerous, healthy cells to its cytotoxicity, i.e., side-effects. As described above, cancer cells are characterized by upregulated incorporation of uracil into DNA, suggesting high expression levels of the relevant enzymes such as OPRT (7). Therefore, cancer cells are more susceptible to 5-FU compared with normal cells because of the augmented RNA dysfunction. Although no data have been available on the relation between OPRT expression level of non-cancerous, healthy cells in cancer patients receiving 5-FU chemotherapy and the patient survival period, it is speculated that the low expression level of OPRT in

Fig. 4 Effect of overexpression (A) and knockdown (B) of OPRT to doxorubicin (DOX) and 5-FU exposure. HeLa cells transfected with full length OPRT/pAcGFP1 or pAcGFP1 vector (A) and OPRT siRNA (siOPRT) or control siRNA (si-Cont) (B) were treated with DOX (0.2 µg/mL) or 5-FU (5 µM) for 24 h. Immunoblot analysis (20 µg protein/lane) using anti-AcGFP1 and anti-cleaved PARP antibodies. Immunoreactive bands of cleaved PARP were quantified by densitometry and normalized to the value of sample transfected with control vector pAcGFP1 after DOX or 5-FU treatment. Anti-β-actin antibody was used as a control. A representative result of three repeated experiments is shown.

the entire body renders patients vulnerable to 5-FU chemotherapy attributable to DNA dysfunction, which might significantly affect the patient survival. Further studies must be conducted to elucidate this point.

The subcellular site for pyrimidine synthesis, including several enzymes mentioned above, has been unclear in mammals. In this regard, we show for the first time that OPRT, one key enzyme in pyrimidine synthesis and the first rate-limiting step to elicit anti-cancer effects of 5-FU, localizes to the Golgi complex by immunocytochemistry. This finding sug-

gests that the Golgi complex is the site for UMP synthesis. A previous report has described that the active site of UDPase, which also generates UMP by UDP hydrolysis, is on the luminal side of the Golgi complex (17). Because rat liver UDPase is involved in protein and lipid glycosylation in the Golgi complex (2), this organelle serves as a center for the metabolism of UMP, which is further phosphorylated to UTP, a substrate for RNA synthesis and an activator of substances in metabolic reaction, such as UDP-glucose. It remains unclear, however, how OPRT is targeted to the Golgi complex, since it contains no apparent consensus motif or signal to this organelle. Taken together, it is conceivable that variable levels of OPRT expression affect chemosensitivity to anti-cancer drug 5-FU and, plausibly, glycosylation of lipid and protein through activated glucose. Our findings warrant further investigation to elucidate the functional implications of OPRT.

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