A diagnostic potential of altered transporter activity for cancer and renal function

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Summary

Membrane transporters play a pivotal role in exchange of substances essential for nutrient homeostasis in humans. Previously many studies revealed interactions between drug transporters and clinically important drugs, suggesting that an altered expression or function of transporters between normal and pathological tissues can be utilized for drug delivery or diagnosis. In the Chapter II, up-regulation of peptide transporter in human cancer cells were targeted to deliver [^{11}C]Gly-Sar as a PET tracer for detection of malignant tissues by planar positron imaging system. Since intracellular accumulation of the tracer via PEPTs specific to xenografts in mice, which were originated from several different human cancer cell lines, was successfully visualized in PET under given conditions, this approach was demonstrated to be promising to diagnose cancer *in vivo*. [^{11}C]Gly-Sar is also superior to [^{18}F]FDG for distinguishing between tumors and inflammatory tissues. In the Chapter III, alteration in expression of several transporters in renal proximal tubules are focused on as a mechanism of regulation of serum L-citrulline levels, which is elevated as kidneys are damaged. Among several candidates including transporters for amino acids and organic anions, Oat1 was identified to predominantly contribute to renal secretion of L-citrulline. Although correlation is not known yet between OAT1-mediated L-citrulline transport and degree of renal failures in humans, OAT1 may be one of important molecular determinants because its metabolisms are not affected under such disease. This thesis experimentally demonstrates possibilities that alteration in transport systems in disease provides important clues for a novel and efficient diagnostic marker to improve quality of life of patients.

Abstract

Plasma membrane transporters are membrane proteins that move their substrate molecules across the plasma membranes of the cells. Considerations of their function are crucial issues not only in the field of pharmacokinetics but also in the field of toxicology and pharmacology, because transporters play a pivotal role in determining absorption, distribution, metabolism, excretion and toxicity of drugs and endogenous compounds. Modulation of the transporter functions could alter disposition, efficacy or, toxicity of certain drugs, as well as modifying drug-drug, or -food interactions. A genetic polymorphism of drug transporters is also an important factor to alter the transport activity. Indeed, single nucleotide polymorphisms have been intensively studied, and led us to many significant findings in clinical. Hence, in this thesis, I focused on the altered expression and/or function of specific transporters under pathological condition, and studied feasibility of transporters as molecular target of specific disease state, as one of a next discipline of transporter studies.

In the Chapter II, a usefulness of peptide transporter to detect malignant tumor tissues is investigated *in vivo* mouse models. Currently, cancer is the leading cause of deaths in nearly all over the world including Japan. Cancer detection in medical exams represents one of the most promising approaches to reducing the growing cancer burden. Positron emission tomography (PET) imaging has emerged as a clinical cornerstone in detection of neoplasmasms, and (re)staging for a number of malignancies because PET imaging has a high intrinsic sensitivity, unlimited depth penetration, and the additional advantages of being fully quantitative and providing higher spatial resolution than single photon emission tomography (SPECT) in the monitoring of functional activity of the tumors. Our research group has previously reported that a peptide transport activity enhanced in the human fibrosarcoma HT1080, compared to that in normal human embryonic fibroblasts IMR-90, implying that peptide transporter is a target for detection of cancer. My ultimate aim of the study is to establish a novel tumor-imaging technology using a PET tracer targeted to peptide transporters overexpressed in human cancer. Since PET probe for peptide transporter, [^{11}C]glycylsarcosine ([^{11}C]Gly-Sar), was reported recently, I hypothesized that cancers expressing peptide transporter could be detected by utilizing [^{11}C]Gly-Sar. All the three human cancer tumor xenografts were well visualized with the planar positron imaging system (PPIS) after injection of [^{11}C]Gly-Sar. Expression of PEPT1 and PEPT2 in those xenografts was confirmed by immunohistochemical analysis. Tumor-to-blood concentration ratios of [^{11}C]Gly-Sar increased in a time-dependent manner and were much higher than unity. Most of the radioactivity found in the tumor tissues was detected as the intact tracer. These results

indicate that [^{11}C]Gly-Sar was taken up by the PEPTx in tumor xenografts. It is noteworthy that [^{11}C]Gly-Sar was minimally accumulated in inflammatory tissues that expressed no PEPT1 or PEPT2 protein, whereas ^{18}F-FDG was highly accumulated, with the values of the selectivity index being .25.1 and 0.72 for [^{11}C]Gly-Sar and [^{18}F]FDG, respectively, suggesting that [^{11}C]Gly-Sar is more selective tracer to detect cancer. The mRNA expression of PEPT1 and PEPT2 were detected by real-time RT-PCR in 27.6% and 93.1%, respectively, of the 60 cancer cell lines examined in this thesis. These results first demonstrate that [^{11}C]Gly-Sar is not only a promising tumor-imaging PET agent but also more efficient to distinguish tumors from inflammatory tissues than [^{18}F]FDG. Since PEPTx was often ubiquitously expressed in various types of tumor cells examined, [^{11}C]Gly-Sar could be useful to detect various types of human cancers (**Chapter II-1**).

Although functional expression of peptide transporter (PEPT1 and PEPT2) in human cancers including human pancreatic cancer cell line AsPC-1 was reported, little is known about physiological meanings of overexpression of peptide transporters in cancer cells. In the later part of this chapter, I hypothesized that peptide transporters are promising molecular targets to inhibit or limit tumor cell growth. I examined effect of inhibition of the transporters by Gly-Sar and other artificial inhibitors on the growth of human pancreatic cancer AsPC-1 cells. As the transporters were blocked in the presence of Gly-Sar or other artificial inhibitors, proliferation of human pancreatic cancer AsPC-1 cells was markedly reduced in a dose-dependent fashion of inhibitors used. Similarly, a non-transported substrate with high afficnity for PEPT1, Bip(OMe)-Sar, inhibited growth of the cancer cells at lower concentrations than those of Gly-Sar used. In addition, the inhibitory effects of Gly-Sar and BCH, which is a typical inhibitor of system L on cell growth were additive, suggesting that each compound was acting at a distinct locus, i.e. peptide transporter and system L, respectively. These results indicate that peptide transporters at least in part contribute to adoptive cell growth of AsPC-1 cell, providing my hypothesis (**Chapter II-2**).

In Chapter III, I focused on diagnosis of kidney function by monitoring the altered function of transporters. Among several amino acids, interestingly, the plasma level of L-citrulline is remarkably elevated in rats and humans with chronic renal failure. Since the metabolism of L-citrulline was not altered in the situation, it was considered that the change in the plasma L-citrulline level would be largely a result of altered renal transport of L-citrulline in proximal tubular cells (PTC). Therefore, I attempted to clarify the mechanism of renal handling of L-citrulline using rats and *in vitro* expression systems. Two mechanisms were considered in the case of elevation of serum L-citrulline level. One is a decrease and/or reduction of secretion impaired of secretory transporter(s), and the other is an increase and/or activation of reabsorptive transporter(s). First, I tested the basolateral uptake of L-citrulline.

In rat kidney slices, L-citrulline uptake was apparently Na^+-dependent, saturable with K_m 68 μM, and significantly inhibited by anionic (*p*-aminohippuric acid) and cationic (tetraethylthyl ammonium) compounds, but not by probenecid at 1 mM. In studies using transporter-overexpressing cells, affinities of L-citrulline transport activity to human organic anion transporter 1 (OAT1) and rat Oat1 were estimated at K_m values of 238 μM and 373 μM, respectively, while OAT2 and 3, and organic cation transporters OCT1 and 2 did not transport L-citrulline. Based on the relative activity factor method, the contribution of rat Oat1 to the overall uptake of L-citrulline in rat kidney slices was approximately 50 to 70%. Thus, human OAT1 and rat Oat1 appear to be major contributors to renal basolateral uptake of L-citrulline. Hence, impairment of these transporters' activities may enhance plasma L-citrulline as renal failure occurs. Since it was reportedly known that protein levels and mRNA expression of human and rat Oat1 is reduced in chronic renal failure, the fluctuation of plasma L-citrulline level can be explained at least in part by the change of apparent activity of OAT1 (**Chapter III-1**).

Next, I characterized L-citrulline transport across the renal tubular epithelial brush-border membranes. L-Citrulline transport was evaluated using primary cultured rat renal proximal tubular cells as well as human kidney proximal tubular cell line, HK-2. L-Citrulline was transported in a Na^+-dependent manner from the apical side of the both cells cultured on permeable supports with microporous membrane. Kinetic analysis indicated that the transport consists of two distinct Na^+-dependent and one Na^+-independent saturable systems in HK-2 cells. The uptake was competitively inhibited by neutral and cationic, but not anionic amino acids. Relatively large cationic (verapamil and quinidine) and anionic (rifampicin and bromosulfophthalein) compounds inhibited the uptake, but smaller ones (tetraethylthyl ammonium or probenecid, estrone-3-sulfate) did not. In HK-2 cells, mRNA expression of *SLC6A19* and *SLC7A9* (that encodes B^0AT1 and $b^{0,+}AT$, respectively) was detected by RT-PCR. In addition, L-citrulline transport was significantly decreased in HK-2 cells with *SLC6A19* and *SLC7A9* silencing, respectively. Hence, these results suggest that amino acid transporters B^0AT1 and/or $b^{0,+}AT$ are at least in part involved in the apical membrane transport of L-citrulline in renal tubular cells for the reabsorbtion of L-citrulline (**Chapter III-2**). Identification of transport systems in the kidney may provide an important insight to use L-citrulline as biomarker to assess the renal insufficiency and therapeutic efficacy and toxicity of drugs.

学位論文審査結果の要旨

本研究は、病態時におけるトランスポーターの発現量や活性変動に着目し、その診断への応用性について検討したものである。まず着目したのは、がん組織に発現が亢進するペプチドトランスポーターPEPTである。PEPT基質のポジトロン標識体を合成し、担がんマウスにおけるがん検出を行なったところ、[^{11}C]Gly-Sarは膵臓がん、胃がん、前立腺がんを移植したマウスの腫瘍を良好に描出し、in vivoでのがん検出の有用性が示された。一方、炎症部への集積が既存のPETプローブである[^{18}F]FDGに比べ小さく、がん選択性の点で優れていることも示された。さらに、多くのヒトがん細胞株でPEPT1とPEPT2が発現していたことから、PEPTを標的としたがん診断の有用性が示された。第二には、腎機能診断にトランスポーター活性変動を利用することを試みた。腎機能評価は適正な薬物療法を行なう上で重要であるが、現行のクレアチニンでは十分に尿細管活性を評価できないため、腎障害時に血中濃度が顕著に上昇するシトルリンに着目した。本研究により、シトルリンの再吸収には中性・カチオン性アミノ酸トランスポーターが、分泌には有機アニオントランスポーターOAT1が関与することがわかった。以上のような結果を総合的に考えた結果、腎障害時にはOAT1の発現量が低下するため、シトルリンの血中濃度が上昇する可能性を示唆することができた。以上、本研究成果はトランスポーター活性変動に基づく新規病態診断方法の樹立に有益な知見をもたらすものであり、今後の診断技術への貢献が期待される点で高く評価されるので、審査委員会は本論文が博士（薬学）に値すると判定する。