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Review

Role of Klotho in the Pathogenesis of Kidney Disease

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Klotho is a single-pass transmembrane protein that performs biological functions. Membrane-bound Klotho acts as coreceptor for the major phosphatonin, fibroblast growth factor 23 (FGF23), while soluble Klotho functions as an endocrine substance. In addition to being abundantly expressed in the distal nephron, Klotho is present in the lumen of proximal tubules, where it inhibits renal inorganic phosphate (Pi) excretion by modulating Na-coupled Pi transporters via enzymatic glycan modification of the transporter proteins, an effect that is completely independent of its role as the FGF23 coreceptor. Acute kidney injury (AKI) and chronic kidney disease (CKD) are states in which there is a systemic Klotho deficiency, making Klotho a very sensitive biomarker of impaired renal function, and the Klotho deficiency exacerbates the impaired decreases in renal function in both AKI and CKD. Decreases in the vitamin D receptor, Ca-sensor receptor, and Klotho-FGF receptor complex in the parathyroid gland facilitate the development of secondary hyperparathyroidism, soft tissue calcification is a major complication of CKD, and is associated with high mortality. Klotho protects against the soft tissue calcification by at least three mechanisms: phosphaturia, preservation of renal function, and a direct effect on vascular smooth muscle cells by inhibiting phosphate uptake and dedifferentiation. In summary, Klotho is a critical molecule in a wide variety of renal diseases and has great potential as a diagnostic and prognostic biomarker as well as for replacement therapy.

Key Words: Klotho, fibroblast growth factor 23, kidney disease, phosphate, vascular calcification

Introduction

The *klotho* gene, named after a Greek goddess who spins the thread of life, was originally identified as a mutated gene in a mouse strain that inherits a premature-aging syndrome in an autosomal recessive manner[1]. At around 3-4 weeks after birth mice defective in *klotho* gene expression being to develop multiple aging-like phenotypes, including growth retardation, hypogonadotropic hypogonadism, rapid thymus atrophy[2], skin atrophy, sarcopenia, vascular calcification, ostopenia[3], pulmonary emphysema[4], impaired conition[5], a hearing disturbance[6], and motor neuron degeneration[7], and they die at around 2 months of age. By contrast, transgenic mice that overexpress Klotho live longer than wild-type mice[8]. Thus, the *klotho* gene may be an aging suppressor gene that extends the life span when overexpressed and accelerates aging when disrupted[9].

The *klotho* gene encodes a single-pass transmembrane protein that belongs to a family 1 glycosidase[10] and is primarily expressed in the renal tubules in the kidney and in the choroids plexus in the brain[1]. In addition to its transmembrane form, Klotho occurs in a soluble secreted form[11)12)] that is derived from an alternatively spliced transcript or cleaved by the Disintegrin and Metalloproteinases (ADAM) family of secretases[13]. Klotho circulates as a soluble protein in the body fluids including blood, urine, and cerebrospinal fluid[14]. The fact that intravenous injection of Klotho regulates inorganic phosphate (Pi) supports its endocrine actions[15]. The above findings suggest that Klotho may function as an endocrine, paracrine, and autocrine substance.

The phenotypes of *klotho*$^{-/-}$ mice[1] and fibroblast growth factor 23 (*Fgf23*)$^{-/-}$ mice[16] are very similar,

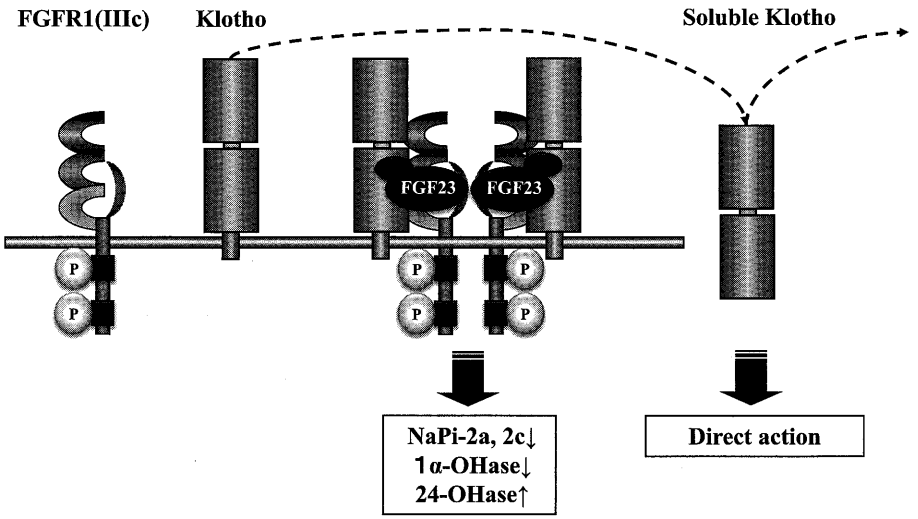

Fig. 1 Direct and indirect actions of Klotho
FGF, fibroblast growth factor; FGFR, FGF receptor; NaPi, sodium-dependent phosphate cotransporter; OHase, hydroxylase (Modified from ref.[20]).

which strongly suggests that the molecules encoded by these genes share a common signaling pathway[17]. As shown in Fig. 1, it is well recognized that membrane Klotho functions as a coreceptor for FGF 23 that amplifies and confers specificity of FGF23 action[18)~21)]. By contrast, soluble Klotho protein functions independently of FGF23[15] and plays an important role in the modulation of ion transporters or channels[22]. Secreted Klotho functions as a humoral factor that regulates activity of multiple glycoproteins on the cell surface, including ion channels and growth factor receptors such as insulin/insulin-like growth factor-1 receptors. Potential contribution of these activities of Klotho protein to aging processes has been discussed in a recent review[23]. Even though Klotho seems to be associated with many organ's function, we decided to focus on the role of Klotho in the kidney. The purpose of this article is to review recent progress in our understanding of the regulation of Pi metabolism by the Klotho-FGF23 axis and to discuss the potential role of Klotho in the pathophysiology of kidney disease.

Role of Klotho in Pi Metabolism

Hyperphosphatemia is a prominent feature of *klotho*$^{-/-}$ mice[1], and administration of recombinant Klotho causes the serum Pi level to decrease to within the normal range. *klotho*$^{-/-}$ mice display increased Na-coupled Pi (NaPi) cotransport activity and increased the expression of NaPi-2a and NaPi-2c cotransporter proteins in comparison with wild-type (WT) mice[24]. To better understand how Klotho affects Pi transport by the renal proximal tubule, Hu et al. detected Klotho expression in the proximal convoluted tubule in addition to a stronger expression in the distal convoluted tubule[15]. As shown in Fig. 2, Klotho is found in proximal tubule cells, their brush border, and the urinary lumen, where Pi homeostasis, which provides direct accessibility to the Na-coupled transporters NaPi-2a, NaPi-2c and Pit2[25]. Vitamin D positively regulates the FGF23 gene expression in bone, injection of 1,25-dihydroxyvitamin $(OH)_2D_3$ increases the serum FGF23 concentration[26]. Binding of 1,25-$(OH)_2D_3$ to the nuclear vitamin D receptor (VDR) induces heterodimerization with another nuclear receptor, RXR, and the VDR-RXR heterodimer binds to the promoter region of the FGF23 gene and transactivates its expression. FGF 23 secreted by osteocytes reaches the Klotho-FGF receptor (FGFR) complex in the kidney and transmits signals that suppress the synthesis and promote the inactivation of 1,25-$(OH)_2D_3$, thereby closing a negative feedback loop (Fig. 2).

Transgenic Klotho-overexpressing mice (*Tg-Klotho*) have low serum Pi level, and their renal fractional excretion of Pi (FE_{phos}) is increased, indicating a renal leak of Pi[15]. Injection of soluble Klotho significantly

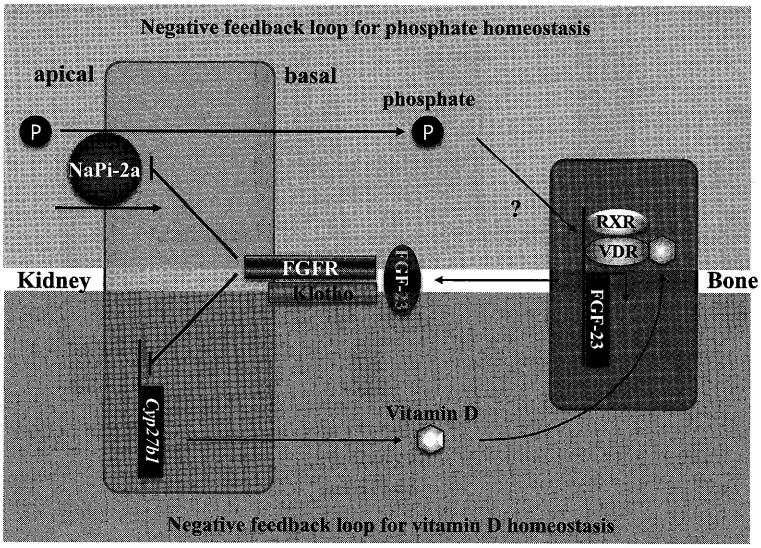

Fig. 2 The bone-kidney axis that regulates phosphate and vitamin D homeostasis
NaPi, sodium-dependent phosphate co-transporter; FGFR, fibroblast growth factor receptor; VDR, vitamin D receptor; RXR, retinoid X receptor; Cyp27b1, cholesterol 27-β hydroxylase (Modified from ref.[23])

increases FE_{phos} and decreases the blood Pi level in normal rats. This phosphaturic action is FGF 23-independent, because Klotho protein efficiently induces phosphaturia and leads to hypophosphatemia even in *Fgf23*$^{-/-}$ mice[15]. The high FE_{phos} is proximal in origin, because the Pi flux significantly decreased in *Tg-Klotho mice* in comparison with WT mice, when a microdissected single proximal convoluted tubule was microperfused in vitro[15]. The direct action of Klotho was further demonstrated in a kidney proximal tubule cell line by addition of Klotho in the absence of FGF23[15]. Klotho has also been found to inhibit NaPi cotransport activity in brush border membrane vesicles, a cell-free system.

The extracellular domain of Klotho contains 2 tandem repeats that have 20-40% amino acid identity with members of the glycosidase family, including β-glucosidase, and it has β-glucuronidase-like enzymatic activity[27]. NaPi-2a is a glycosylated protein[28]. Direct inhibition of NaPi transport by Klotho is mimicked by recombinant β-glucuronidase but not by sialidase[15]. The inhibitory effect of Klotho on NaPi transport is blocked by the β-glucuronidase inhibitor, D-saccharic acid-1,4-lactone but not by the sialidase inhibitor, deoxy-N-acetyl-neuraminic acid. This puts glucoronate removal as a key mechanism and raises the question of how this glycan modification alters transport activity. Klotho shifts NaPi-2a from the full-length form to smaller peptides, and deglycosylation of NaPi-2a facilitates its proteolysis[15]. Protease inhibitors prevent the proteolysis, but do not reverse the Klotho-induced inhibition of transport. These findings indicate that Klotho-induced deglycosylation is sufficient to suppress Na-dependent Pi transport and that subsequent proteolysis is not required. Thus, Klotho modulates NaPi-2a in a biphasic fashion by two distinct mechanisms.

Hyperphosphatemia is universally observed in chronic kidney disease (CKD) patients[29] and is a potent predictor of cardiovascular morbidity and mortality[30]. Controlling serum Pi by restriction of intake[31] and treatment with a phosphate binder[32][33] improves the clinical outcome of CKD patients. Lack of the phosphaturic action of Klotho protein is an important pathogenic factor in CKD.

Klotho Deficiency and Kidney Disease

Klotho has been shown to be a cytoprotective protein that protects against oxidative stress and ischemia-reperfusion injury (IRI)[34]. We have reported finding that Klotho protein and transcript levels in kidneys and kidney cell lines are decreased by exposure to oxidative stress or IRI[35][36]. Increasing evidence suggests a relationship between oxidative stress and kidney disease[37]~[40]. Levels of Klotho

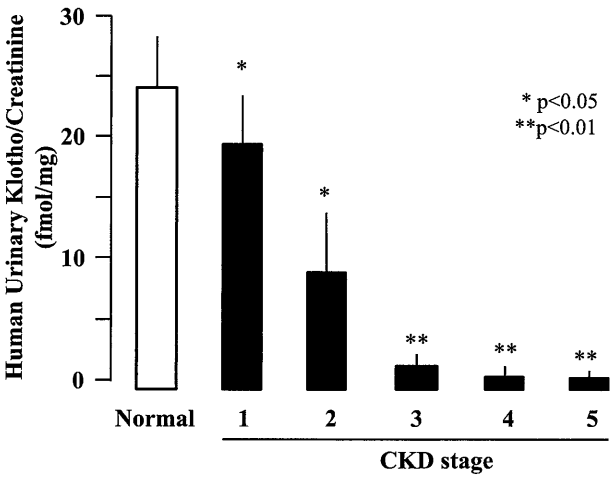

Fig. 3 Urinary Klotho excretion by chronic kidney disease (CKD) patients[48]

protein and transcripts are decreased in kidneys and kidney cell lines by angiotensin II infusion[41][42] and hypertension[43]. In addition, renal Klotho decreased in CKD in humans[44] and in several experimental animal models of CKD[45][46]. Since the kidney is the major organ in which Klotho is expressed, perhaps it is not surprising that Klotho expression is decreased when the kidney is severely diseased. The question is whether there is endocrine Klotho deficiency that can potentially have far-reaching effects. Until recently, there have been no data on blood Klotho in acute or/and chronic kidney damage.

Two recent studies reported the blood Klotho levels of rodents[47][48]. After acute kidney injury (AKI) rodents develop rapid, severe reductions in Klotho levels in the blood, kidney, and urine, but this reduction is fully reversible upon recovery of kidney function, indicating that AKI is a transient state of endocrine Klotho deficiency[47]. The mechanism of the Klotho down-regulation in AKI is not known. The rapid, severe decrease may not stem only from reduction of Klotho mRNA, as Klotho transcripts are only down-regulated to 50% of baseline[47]. In addition, Klotho down-regulation occurs before changes in other markers of kidney damage[47]. Oxidative stress has been shown to cause decreases in Klotho mRNA and protein in a cultured cell line[49]. Tumor necrosis factor (TNF) and interferon-γ (IFN-γ) reduce renal Klotho mRNA and protein[50]. Whether the increased TNF and IFN-γ levels in AKI lead to Klotho down-regulation remains to be investigated[51].

End-stage CKD patients[44] and animals[52] have reduced Klotho levels in their kidneys, but there were no data on their blood or urine Klotho levels until a recent study showed very low blood, kidney and urine Klotho levels in a murine model of CKD and the investigators postulated that CKD is a state of "pan-deficiency" of Klotho[48]. That study measured urinary Klotho in CKD patients as a surrogate and found that CKD patients have reduced urinary Klotho levels (Fig. 3). Klotho deficiency occurs as early as stage 1 and 2 CKD, and the degree of the decreases in both rodent and human CKD are closely correlated with the size of decreases in estimated glomerular filtration rate. Thus, the urinary Klotho concentration is an extremely sensitive and early marker of CKD, and its decline parallels the loss of kidney function[48]. The mechanism of the Klotho down-regulation in AKI and CKD needs to be determined, because may generate treatment modalities to restore endogenous Klotho expression.

Renoprotective Effects of Klotho

Based on experimental and human data, Klotho levels are closely correlated to kidney function, suggesting that Klotho is a useful novel biomarker for presence of renal disease. However, the fundamental question of enormous biologic and clinical significance is whether Klotho deficiency contributes to the pathogenesis and complications of kidney disease. Hu et al[47][48] induced AKI and CKD in mice that had different Klotho levels: low Klotho levels (heterozygous Klotho haplodeficiency, *klotho*$^{+/-}$), normal (WT), and high Klotho levels (transgenic overexpression of Klotho, *Tg-Klotho*). In the AKI model, the *klotho*$^{+/-}$ mice had low Klotho protein levels in plasma, kidney, and urine at baseline, and they became undetectable after the AKI induction, as they developed more severe renal dysfunction. Conversely, the *Tg-Klotho* mice had high renal, plasma, and urinary Klotho levels at baseline, and were more resistant to an IRI insult than WT AKI mice. These findings indicate that Klotho deficiency exacerbates and Klotho overexpression ameliorates rodent AKI, suggesting that Klotho is useful as a

biomarker. We have demonstrated a protective effect of Klotho on AKI using adenovirus delivery of the *klotho* gene before IRI[49], whereas Hu et al. administered recombinant Klotho protein 30-60 minutes after the IRI and demonstrated reduction of histologic damage and impaired function[47]. This evidence is of more practical value, because practitioners are rarely able to interfere with AKI prior to the insult. The prospect of using Klotho is to reduce kidney damage and promote kidney recovery. The mechanisms by which Klotho protects the kidney from injury are unknown, but may include antiapoptosis and antisenescence[53].

It is also necessary to examine the relationship between Klotho deficiency and the pathogenesis of CKD. The results of studies of different animal models of CKD performed in several laboratories support a beneficial effect of Klotho on CKD. Viral delivery of the *klotho* gene leads to better maintenance of kidney function, a decrease in urinary protein, and amelioration of the tubulointerstitial changes induced by chronic angiotensin II infusion[41]. Viral delivery of Klotho decreases blood pressure, improves kidney histology, and inhibits oxidation in spontaneously hypertensive rats[43]. Moreover, restoration of Klotho in immune-mediated glomerulonephritis by transfection with an overexpressing *klotho* gene has been shown to suppress oxidation, decrease kidney damage, and increase survival[46]. Another study used a model of CKD created by uninephrectomy plus contralateral IRI and demonstrated hypertension, anemia, decreased creatinine clearance, increased proteinuria, and much more severe interstitial fibrosis and glomerular sclerosis in *klotho*$^{+/-}$ CKD mice in comparison with WT CKD mice. Conversely, all of these changes were much less severe in the *Tg-Klotho* CKD mice[15]. Thus, Klotho has been shown to be a renoprotective agent in animal models of CKD.

Relation Between Klotho and Soft Tissue Calcification in CKD

Hyperphosphatemia accelerates complications of CKD, such as hyperparathyroidism and cardiovascular calcification[54], and control of the blood Pi concentration ameliorates these serious complications[55]. The decrease in serum calcium (Ca) in CKD is crucial to the development of secondary hyperparathyroidism and is secondary to Pi retention and low levels of 1,25-$(OH)_2D_3$. Pi retention increases FGF23 secretion, which in conjunction with its cofactor, Klotho, decreases the activity of the 1α-hydroxylase and increases the activity of the 24 α-hydroxylase, which, in turn, results in a decrease in the level of circulating 1,25-$(OH)_2D_3$. In addition, Pi retention posttranscriptionally increases the synthesis of parathyroid hormone (PTH), independent of the decrease in Ca, and 1,25-$(OH)_2D_3$, suppresses transcription of the PTH gene, independent of Ca. Decreases in the VDR, Ca-sensor receptor, and Klotho-FGFR complex in the parathyroid gland accelerate the development of secondary hyperparathyroidism (Fig. 4)[56].

The fact that the CKD-associated vascular calcification observed in *Klotho*$^{-/-}$ and WT mice is completely prevented in *Tg-Klotho* mice supports the paradigm that Klotho suppresses ectopic calcification[8]. While there is severe calcification in the several organs of both WT and *Klotho*$^{+/-}$ CKD mice, there is very little or no calcification in the organs of *Tg-Klotho* CKD animals[48]. The Ca content of the aortas and kidneys of both WT and *klotho*$^{+/-}$ CKD mice is higher than sham. This increase is ameliorated by overexpression of Klotho[48]. The elevation of PTH in WT CKD mice is blunted by Klotho overexpression and aggravated by Klotho deficiency.

One determinant of soft tissue calcification is the plasma Pi concentration. Both *Klotho*$^{+/-}$ and WT animals with CKD have high blood Pi levels. By contrast, *Tg-Klotho* CKD mice show less hyperphosphatemia[48]. Since Klotho is a potent phosphaturic substance, a second mechanism by which Klotho decreases soft tissue calcification is by the lowering plasma Pi levels by promoting phosphaturia.

The Na-coupled Pi transporters Pit1 and Pit2 are key modulators of the Pi influx into vascular smooth muscle cells (VSMCs) and play a pathogenic role in vascular calcification[57)~59]. Up-regulation of *Runx2*, a marker of an osteoblast-like phenotype, and down-regulation of *SM22*, a marker of contractile smooth muscle cells, are typically seen in vascular calcifica-

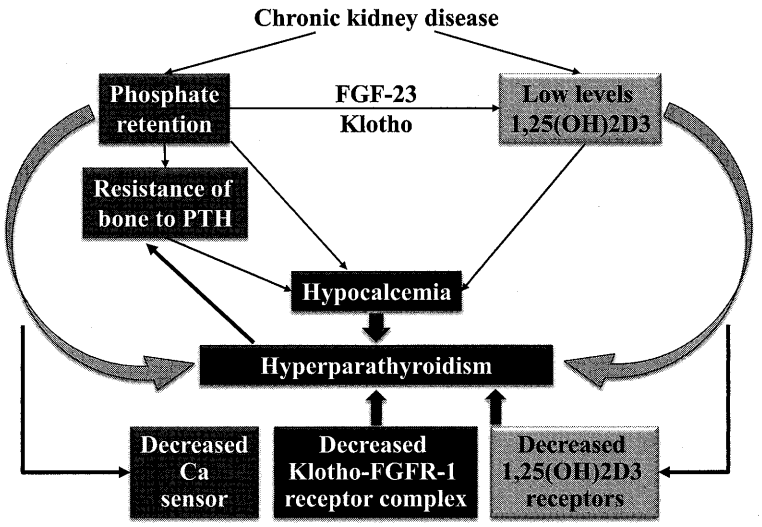

Fig. 4 Factors involved in the pathogenesis of secondary hyperparathyroidism[56].

tion[59]. *Pit1*, *Pit2* and *Runx2* mRNAs are increased and *SM22* is decreased in *klotho*$^{-/-}$ mice, while overexpression of Klotho has the opposite effect. Klotho may control the balance between differentiation and dedifferentiation of VSMCs[48]. CKD induces a pattern similar to the pattern senn in Klotho deficiency, and Klotho overexpression has been found to completely block the changes induced by CKD. When VSMCs are grown in vitro, Klotho inhibits the Na-dependent Pi influx and minimizes the mineralization induced by high ambient Pi[48]. Taken together, the above findings indicate that Klotho exerts its anticalcification effect by at least three mechanisms: phosphaturia, preservation of kidney function, and a direct effect on vascular smooth muscle.

Conclusion

AKI is followed by a transient, severe renal and endocrine Klotho deficiency, while CKD is a sustained state of systemic Klotho deficiency. Klotho is not merely a sensitive early biomarker of kidney disease, but plays a pathogenic role in the progression of kidney disease, disturbed mineral metabolism, and vascular calcification in CKD. Early administration of exogenous Klotho protein or stimulation of expression of endogenous Klotho may improve kidney function in both AKI and CKD. The potential utility of Klotho in clinical practice is at least twofold. First, Klotho could serve as a sensitive early biomarker of kidney disease. Second, Klotho supplementation may provide a novel therapy for AKI by limiting damage and promoting recovery, and for CKD by slowing progression and preventing and reversing complications.

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腎臓病の病態における Klotho の役割

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Klotho は生物学的活性を有する一回膜貫通型の蛋白である．膜結合型の Klotho は，主要なリン調節因子である fibroblast growth factor 23（FGF23）の受容体として働き，可溶型は内分泌物質として作用している．腎の遠位尿細管に多く発現しているが，近位尿細管の管腔側にも存在する．近位尿細管においては，ナトリウム依存性リン共輸送体を介して，負のリン排泄調節を行っている．この作用は，FGF23 と完全に独立した系として機能している．急性腎障害や慢性腎臓病（CKD）は，全身的な Klotho の欠乏状態であり，Klotho が腎障害の程度を予測する鋭敏なバイオマーカーであることを示唆する．CKD においては，副甲状腺におけるビタミン D 受容体，カルシウム感受性受容体および Klotho-FGF23 複合体の発現低下が，二次性副甲状腺機能亢進症の進展に関与している．また，Klotho は軟部組織の石灰化を抑制する作用を有するが，リン排泄の調節，腎機能の保持および平滑筋細胞への直接作用など，少なくとも 3 つの機序が考えられている．これらのことから，Klotho は腎臓病の病態における鍵を握る分子として注目されており，腎障害のバイオマーカーおよび治療薬としての可能性を秘めている．