Cardiovascular Regeneration Therapies Using Tissue Engineering Approaches

The fact that the cardiovascular system transports oxygen and nutrients to all parts of the body makes any threat to its well-being a serious hazard to organs, tissues, and cells. This fundamental truth underlines the importance of research into cardiovascular regeneration and, therefore, of this collection outlining the latest developments in the field. With input from many of the leading researchers and practitioners, this book describes techniques for therapeutic angiogenesis and vasculogenesis for the treatment of ischemic diseases and examines the current approaches to angiogenic cytokines, cardiovascular stem cells, and tissue-engineering tools. The latest clinical results using bone marrow-derived mononuclear cell therapy for intractable circulatory disorders are also covered. This body of work will be a valuable resource for professionals in the fields of cardiovascular surgery, regenerative medicine, and tissue engineering.

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TWO FUNDAMENTAL REGULATORY FACTORS OF THE Na^+/H^+ EXCHANGERS *THE PROTON AND CHP*

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1. INTRODUCTION

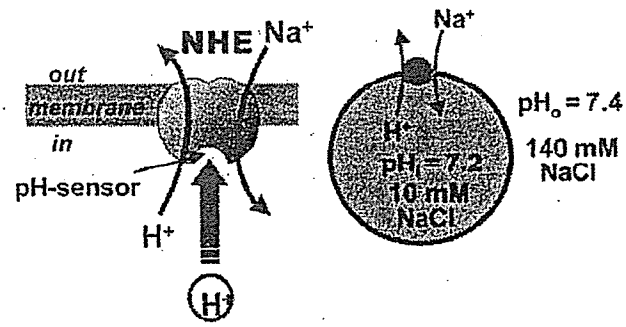
The Na^+/H^+ exchangers (NHEs) are plasma membrane transporters that regulate pH homeostasis, cell volume, and transepithelial Na^+ absorption (1-4). At present, the NHE family includes eight isoforms (NHE1-NHE8) that differ in their tissue and subcellular localizations. An intriguing feature of exchangers is that their activities are controlled by various extrinsic factors, including hormones, growth factors, pharmacological agents, and mechanical stimuli (1-4). The regulatory mechanisms of NHE1 and NHE3 isoforms have been studied extensively. For example, NHE1 has been reported to occur via the involvement of a variety of signaling molecules, i.e., calcinuerin B–homologous protein (CHP) (5,6), Ca^{2+}/calmodulin (7,8), 14-3-3 protein (9), Nck-interacting kinase (NIK) (10), and phosphatidylinositol 4,5-bisphosphate (11). Although the interrelationships among these molecules are not known, several transmit signals to the more general NHE1 regulator, the intracellular proton, because many extracellular stimuli control NHE1 activity by changing the apparent affinity for intracellular H^+, probably at the level of the allosteric "H^+-modifier site" (12, and see references 1-4 for review). Therefore, the cytosolic proton is itself an important regulatory factor. Furthermore, we recently have found that CHP is an essential cofactor for supporting the physiological activity of plasma membrane exchangers (6). In this chapter, we focus on two important cytosolic factors, the proton and

CHP. Our recent results show that these two factors are indispensable for the normal function of these exchangers under physiological conditions. In addition, we focus on the role of CHP2 (13), another isoform of CHP.

2. THE PROTON

2.1 The H^+-Modifier Site

In cells, intracellular pH (pH_i) is usually set at a much lower value (~7.2) than that expected from the thermodynamic equilibrium of Na^+ and H^+ (greater than pH 8.0; Figure 1). This "set point" behavior has been attributed to the existence of an allosteric regulatory site(s), often called the "H^+-modifier" site or "pH-sensor." Twenty years ago, Aronson et al. (12) presented evidence for the existence of such a site, based on elegant experiments with renal brush border membrane vesicles. Their data led to the idea that an exchanger becomes active only when a proton occupies the cytosolic H^+-modifier site. Intracellular H^+ has subsequently been shown to activate Na^+/H^+ exchange in several experimental systems (see refs. 1-4 for reviews), including various types of cells expressing endogenous or exogenous exchangers. The H^+-modifier site appears to play several important physiological roles in the regulation of pH_i by the Na^+/H^+ exchanger (Figure 1). First, dissociation of H^+ from the modifier site completely prevents exchange activity and thereby protects cells from alkalosis. Second, H^+ occupation of the modifier site markedly stimulates



Physiological Role of pH-Sensor

1. Protection from alkalosis
2. Recovery from acidosis
3. Modulation of activity

Figure 1. Physiological role of the pH-sensor. The H^+-modifier site (pH-sensor) is different from the H^+-transport site. The pH-sensor plays a critical role in regulation of pH_i.

exchange activity and thereby permits rapid recovery of cells from acidosis. Third, many extrinsic stimuli are capable of delicately modulating the exchange activity in the neutral pH_i range by changing the affinity of H^+ for the modifier site.

Many studies with native or NHE-transfected cells have revealed that, in contrast to the simple kinetics of external Na^+ and H^+, exchange activity obeys complex cooperative kinetics of internal H^+ that involve at least two H^+ binding sites. However, because only the forward mode of exchange was measured in these studies, whether such a regulatory site exists in the various NHE isoforms is not certain. In addition, it was difficult in these studies to distinguish the putative H^+-modifier and H^+-transport sites. Recently, we have tried to dissect kinetically these two H^+-binding sites by measuring EIPA-sensitive $^{22}Na^+$ efflux from cells, i.e., the reverse mode of exchange. We loaded $^{22}Na^+$ into NHE1 transfectants and at the same time clamped pH_i at various values by means of the K^+/nigericin technique. After removing the radioactive preincubation solution, we added a Na^+-free non-radioactive solution (pH 7.4) to start $^{22}Na^+$ efflux from the cells. We observed that intracellular acidification dramatically stimulates $^{22}Na^+$ efflux through NHE1, whereas alkalinization completely inhibits efflux (unpublished observation). If we assume that the Na^+/H^+ exchange obeys a simple counter-transport reaction, the outward-directed H^+ gradient should inhibit $^{22}Na^+$ efflux. The unexpected stimulation of $^{22}Na^+$ efflux by the intracellular proton is consistent with the idea that the exchange activity is regulated by protonation/deprotonation at an H^+-modifier site(s) that is different from the H^+-transport site. The $^{22}Na^+$ efflux of the NHE1 transfectants exhibited a bell-shaped pH_i dependence (unpublished observations, see Figure 2). The activation phase of $^{22}Na^+$ efflux caused by H^+ is very steep in the pH_i range of 7.0 to 7.5, suggesting that two or more protons are involved in exchanger activation. In contrast, strong acidification ($<pH_i$ 6.2) inhibited $^{22}Na^+$ efflux. Presumably, this inhibition results from competition between intracellular H^+ and Na^+ at the transport site. Thus, measurement of $^{22}Na^+$ efflux allowed us to evaluate separately the H^+-modifier and H^+-transport sites, which are related, respectively, to the descending and ascending slopes of the pH_i dependence curve. In fact, the bell-shaped pH_i profile of $^{22}Na^+$ efflux is adequately explained by assuming the binding of multiple protons at the modifier site or sites and a single proton at the transport site. The steep pH_i profile of $^{22}Na^+$ efflux in the neutral pH_i range (see Figure 2) suggests that the modifier site of NHE1 has properties that permit the physiological roles described in Figure 1. However, it should be noted that the complex kinetic behavior of H^+-induced activation has been reported previously: i) transient kinetic studies of membrane vesicles have revealed that the external Na^+

dependence becomes cooperative when the inside of vesicles is acid-loaded (14), suggesting that protonation of the modifier site may change the

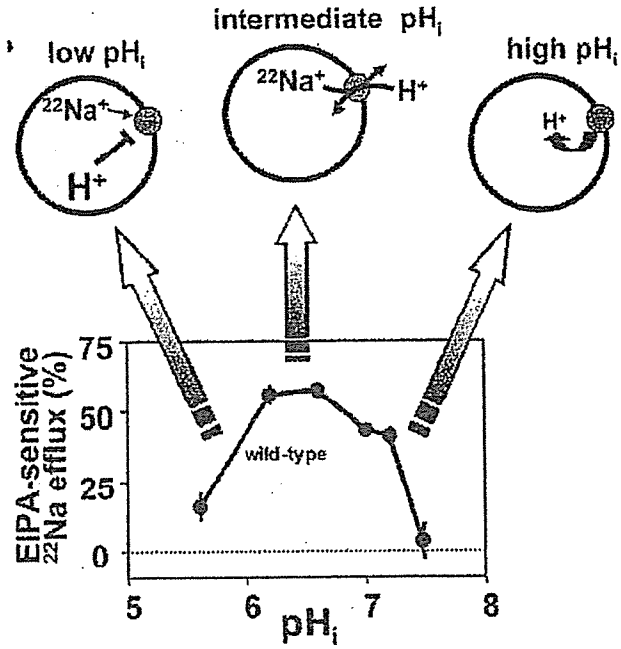


Figure 2. The pH_i profile of $^{22}Na^+$ efflux from NHE1 transfectants. Plotting EIPA-sensitive $^{22}Na^+$ efflux during the initial 3 min indicates that $^{22}Na^+$ efflux is dramatically activated by a slight cytosolic acidification (pH_i 7.0-7.2), reaches a maximum at pH_i 6.6, and then decreases with decreasing pH_i (5.6-6.2). Inhibition by cell alkalinization appears to be due to deprotonation at the modifier site, whereas inhibition by cell acidification appears to be due to competition between H^+ and Na^+ at the transport site.

oligomeric interaction; ii) NHE3, unlike NHE1, is slowly (~5 min) activated by intracellular acidification (15), suggesting that a slow conformational change of NHE3 may be involved; iii) the pH_i and pH_o dependencies of the forward mode of exchange are mutually interrelated (for example, pH_i dependence becomes more acidic with decreasing pH_o [16]), suggesting that the inhibitory interaction of external H^+ with the exchanger may somehow change the properties of the intracellular H^+-modifier site; and iv) intracellular Na^+ is able to activate the exchanger (17), suggesting the possible interaction of Na^+ with the H^+-modifier site. All these data suggest that the H^+-induced activation of the exchangers is regulated by a complex mechanism involving multiple residues and dynamic conformational changes, rather than a simple mechanism such as protonation of ionizable residue(s).

2.2 Critical Regions Involved In pH-Sensing

All NHE molecules can be separated into two large moieties, the amino-terminal transmembrane domain and the carboxyl-terminal hydrophilic domain. On the basis of a cysteine-accessibility analysis, we recently presented a new topology model for NHE1 that consists of 12 membrane-spanning segments with their N- and C-tails in the cytosol (18) (see Figure 3A). Whereas, the N-terminal transmembrane domain exerts the Na^+/H^+ exchange reaction, the cytoplasmic domain functions as the regulatory domain. The initial deletion mutant study (19) provided evidence that the C-terminal cytoplasmic domain plays a critical role in regulating the pH sensing by NHE1. Deletion of the complete cytoplasmic domain resulted in a large acidic shift in the pH_i dependence of the Na^+/H^+ exchange, suggesting that the cytoplasmic domain plays a critical role in pH sensing. Furthermore, the cytoplasmic domain was found to be separated into at least four distinct domains in terms of pH_i sensitivity (20). Deletion of subdomain I (aa 503-595) results in a reduction of pH_i sensitivity, whereas deletion of subdomain III (aa 635-656) results in an enhancement of pH_i sensitivity (Figure 3A). Thus, the former subdomain plays a role in maintaining pH_i sensitivity, as well as in exerting various regulatory functions, such as growth factor–induced activation and ATP depletion–induced inhibition (20). On the other hand, the latter subdomain functions as an autoinhibitory domain and may be involved in Ca^{2+}-induced activation as a Ca^{2+}/calmodulin-binding domain (7,8).

In contrast to the C-terminal cytoplasmic domain, the important region within the N-terminal transmembrane domain that is involved in pH_i sensitivity has not been identified so far. Histidine residues within the transmembrane domain have generally been thought to be good candidates for amino acids involved in the regulation of pH_i sensing, because the imidazole moiety has a pK of 6.2 and is the only amino acid side chain that ionizes within the physiological pH range. Indeed, His^{225} and His^{367} have been identified as important residues for pH sensing by the Na^+/H^+ antiporters of *Escherichia coli* (NhaA) (21) and *Schizosaccharomyces pombe* (Sod2) (22), respectively. However, such residues have not yet been identified in mammalian exchangers. Despite an extensive search for histidine residues involved in pH_i sensitivity, we have failed to identify critical residues within the N-terminal transmembrane domain (S. Wakabayashi, unpublished observation). In addition, His^{35}, His^{120}, and His^{349} within transmembrane segments (TMs) (23) and the cytosolic histidine cluster (HYGHHH) (22) of NHE1 do not appear to be directly involved in the exchange activity. Thus, histidine residues do not appear to

be significantly involved in pH sensing, although we cannot exclude the possibility of the involvement of multiple histidine residues.

Recently, we have focused on the putative 11th transmembrane segment (TM11) and its surrounding regions and found that substituting the Arg^{440} in intracellular loop 5 (IL5) with various residues (Cys, Asp, Glu, His, and Leu) greatly shifts the pH_i dependence of $^{22}Na^+$ uptake to the acidic side, whereas mutations of Gly^{455} and Gly^{456} within the highly conserved glycine-rich region of TM11 significantly shifts it to the alkaline side (unpublished

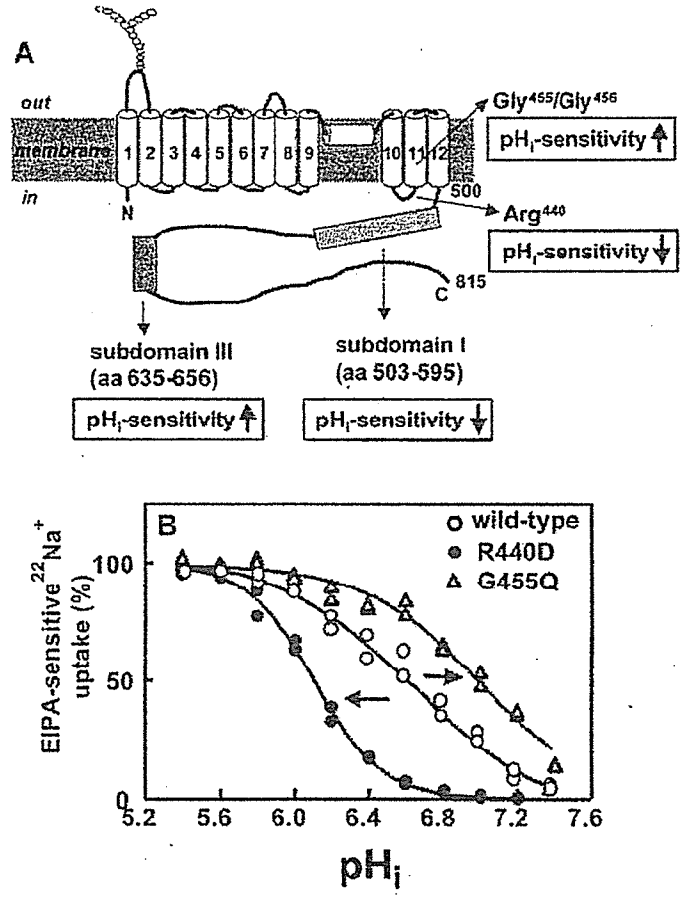


Figure 3. Schematic representation of mutation-sensitive regions involved in pH_i sensing. A, regions or residues for which deletion or point mutations shift the pH_i dependence of exchange toward the acidic or alkaline side. B, pH_i dependencies of EIPA-sensitive $^{22}Na^+$ uptake of cells expressing the mutant NHE1s, R440D or G455Q.

observation, see Figure 3B). Substituting Lys for Arg^{440} did not change pH_i dependence significantly, suggesting that the charge on Arg^{440} is important for pH_i sensing, whereas the alkaline shift that occurred by substituting Gly^{455} with bulky residues such as Gln and Val suggests that a mutation-induced steric hindrance in TM11 may be involved. These two mutant exchangers did not cause changes in the apparent affinities for extracellular

Na^+, H^+, and the inhibitor EIPA, suggesting that the mutations affect a restricted region. Importantly, these mutations shifted the bell-shaped pH_i profile of $^{22}Na^+$ efflux to the acidic (R440D) and the alkaline side (G455Q), respectively. These data suggest that Arg^{440} is an essential residue for regulating pH_i sensing, presumably via a charge-dependent interaction with the cytoplasmic regulatory domain, whereas the Gly residues in TM11 may form a structurally important element for the proper functioning of the putative"pH_i-sensor" of NHE1. Further study is required to clarify how these residues cooperate with the cytoplasmic domain in the regulation of pH sensing.

3. CHP

3.1 CHP1 As An Essential Cofactor

In 1996, Lin and Barber (5) and Kanazawa's group (Osaka U., Japan) have independently identified a novel Ca^{2+}-binding protein, CHP (calcineurin B–homologous protein, here designated CHP1), that interacts with NHE1. CHP1 may be the same protein as the 24-kD NHE1-binding protein identified by another group (24). CHP1 is ubiquitously expressed and is homologous to the calcineurin B subunit (5). CHP1 has four Ca^{2+}-binding motifs (EF-hand), of which two ancestral motifs do not bind Ca^{2+}. The N-terminus of CHP1 is myristoylated. The same protein has been identified independently as a factor (known as p22) required for the vesicular transport of proteins (25). CHP1 has been reported to inhibit calcineurin phosphatase activity (26) and to associate with microtubules (27). CHP1 has been reported to interact with a serine/threonine protein kinase (DRAK2) involved in apoptotic cell death (28) and with a kinesin family member, KIF1Bβ2 (29).

An initial study (5) suggested that CHP1 binds to the cytoplasmic region (aa 566-635) of NHE1. However, on the basis of the *in vitro* pull-down assay, far-Western staining, co-immunoprecipitation, and the observation of co-localization of GFP-tagged CHP1 with NHE1-3, we have shown that CHP1 binds to the juxtamembrane domain (aa 510-530 in the case of NHE1) within the cytoplasmic domain of NHE1-4 (6). The CHP1-binding domain of NHE1 is predicted to form a conserved α-helix similar to that of the calcineurin B–binding domain within the calcineurin A subunit (30). Based on the data for interaction of CHP1 with NHE1 mutant proteins, we have concluded that hydrophobic residues within the CHP1-binding domain are important for interaction, as in the calcineurin A/B complex. Myristoylation and Ca^{2+} binding are not essential for interacting with NHE1. Figure 4

shows fluorescent images of cells expressing the CHP1 fusion protein conjugated with green fluorescent protein (GFP). CHP1 was distributed uniformly in the cytosol of exchanger-deficient PS120 cells (Figure 4). This fusion protein became partly localized in the plasma membrane when exogenous NHE1 was co-expressed (Figure 4B). Thus, NHE1 seems to be the principal target for CHP1 in the membrane.

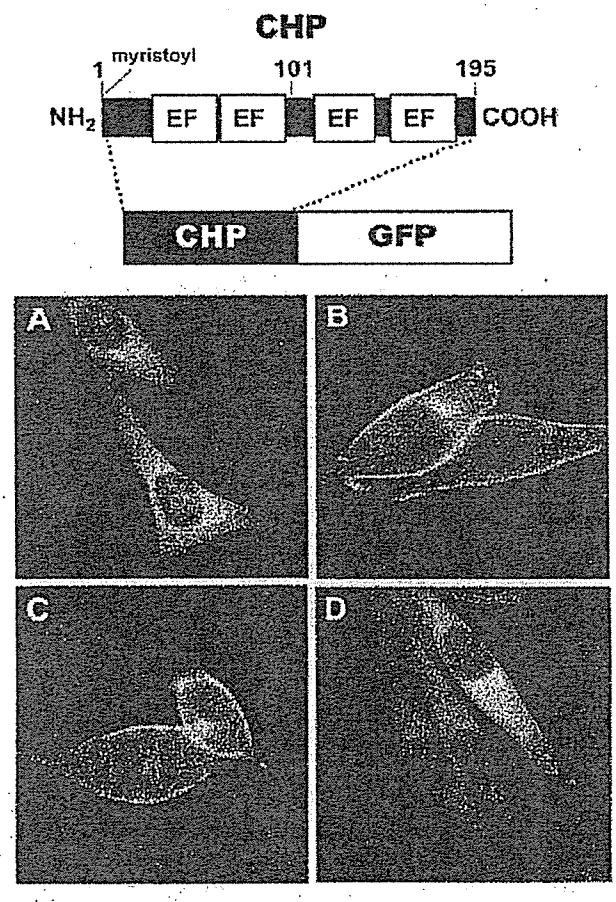


Figure 4. Structure of CHP and subcellular localization of GFP-tagged CHP. CHP1 (B) and CHP2 (C) become localized in the plasma membrane when NHE1 is expressed. A, CHP1-GFP proteins are expressed in exchanger-deficient PS120 cells. D, CHP2-GFP was expressed in cells expressing CHP-binding deficient NHE1 (4R).

Functional analysis revealed the important role of CHP1 in NHE: i) CHP1-binding–deficient mutations of NHE1-3 dramatically reduced exchange activity (V_{max}); and ii) CHP1 depletion by injecting the competitive CHP-binding regions of NHE1 into *Xenopus* oocytes resulted in a dramatic reduction in activity. Thus, CHP1 serves as a common essential cofactor that supports the physiological activity of plasma membrane NHEs.

A single polypeptide for each NHE isoform has generally been considered sufficient for normal exchange activity; however, our data

suggest that multiple exchangers require physical interaction with a common protein, CHP, for expression of the activity.

3.2 Role Of CHP2

Another human CHP isoform (here designated CHP2) has been identified in a human cancer patient (31) (NCBI nucleotide accession number NM022097 with the designation of hepatocellular carcinoma antigen gene 520). CHP2 protein shares a 61% amino acid identity with CHP1. CHP2, like CHP1, contains an N-terminal myristoylation site (Gly2) as well as four EF-hand Ca^{2+}-binding motifs. In contrast to CHP1, however, the expression of CHP2 is extremely low in most human tissues. However, CHP2 was expressed at relatively high levels in malignantly transformed cells, such as hepatoma, colon adenocarcinoma, and leukemia cells, suggesting that CHP2 may be involved in the phenotypic change of NHE properties. Recently, CHP2 was reported to be expressed in the small and large intestine of the rat (32).

We found that i) GFP-tagged CHP2 co-localized with NHEs 1-3, but not with their mutants lacking CHP1-binding ability (see Figure 4); ii) recombinant CHP2 was bound to a MBP fusion protein containing the NHE1 cytoplasmic domain, but not to the fusion protein containing the NHE6 cytoplasmic domain; iii) CHP2 enhanced the exchange activities of NHE1 and NHE3 when co-expressed with them in oocytes; and iv) exchange activity (V_{max}) in cells co-expressing CHP2/NHE1 was comparable with that in cells co-expressing CHP1/NHE1. Therefore, CHP2 competes with CHP1 for binding at the same juxtamembrane domain within the cytoplasmic domain of plasma membrane NHEs and, like CHP1, has the ability to up-regulate the exchange activity. The competition experiment revealed that CHP2 interacts more strongly (5-fold) with NHE1 than does CHP1.

CHP1/NHE1 and CHP2/NHE1 cells responded differently to serum depletion, although they did exhibit a similarly high exchange activity when maintained in serum. The cells co-expressing CHP2/NHE1 but not CHP1/NHE1 exhibited high steady-state levels of pH_i, even in the absence of serum. In addition, exchange activity in CHP2/NHE1 cells was permanently activated in a serum-independent manner. Furthermore, CHP2/NHE1 cells were much more resistant to serum than were CHP1/NHE1 cells (see Figure 5). Surprisingly, 60% of cells expressing CHP2/NHE1 were still viable 10 days after serum starvation, when all CHP1/NHE1 cells had lost viability. The high viability of CHP2/NHE1 cells appears to be due to high pH_i caused by serum-independent activation of NHE1, because cells overexpressing CHP2 were sensitive to serum

starvation when active NHE1 was not expressed or when EIPA was present in the medium.

It is well known that activation of NHE1 is associated with oncogenic transformation (33-36). For example, cells transformed by ras (34, 35) or E7 oncogenes (36) have been shown to maintain a high pH_i in the absence of serum with an accompanying high activity of NHE1, and this response may be one of key factors involved in abnormal cell growth, high resistance to serum deprivation, and abnormal cell invasion. In addition, a high pH_i due to the activation of NHE1 has been observed in various malignantly transformed cells, such as human leukemic (37), malignant glioma (38) and breast cancer cells (39). All these studies suggest that NHE1 becomes permanently activated in many malignant cells; thus, CHP2/NHE1 cells have a property similar to that found in malignantly transformed cells.

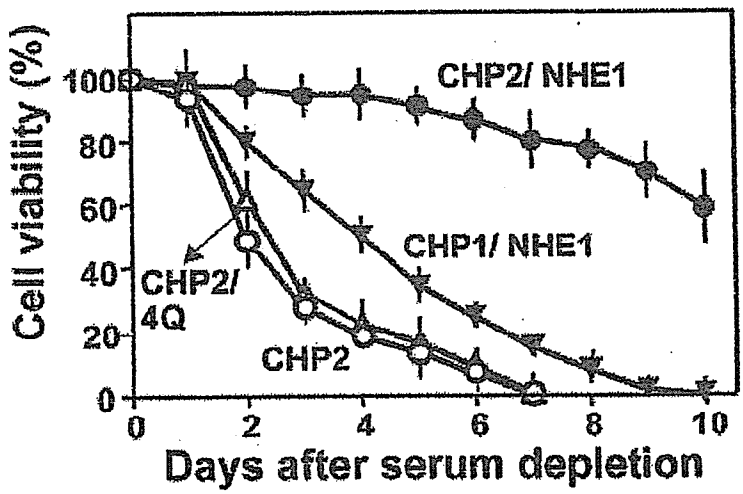


Figure 5. Cells were plated on dishes and serum-depleted on the next day, and the numbers of viable cells remaining were counted on the indicated days. Note the high viability of cells co-expressing CHP2/NHE1, compared with cells co-expressing CHP1/NHE1. Cells expressing only CHP2 or co-expressing CHP2 and CHP-binding–defective mutant 4Q are very sensitive to serum-deprivation.

Figure 6 shows our present working hypothesis. In normal cells, CHP1 interacts with NHE1 at its juxtamembrane region within the cytoplasmic domain. When cells are malignantly transformed, CHP2 becomes expressed at a relatively high level. Because NHE1 interacts with CHP2 more strongly than with CHP1, CHP2 binds predominantly to NHE1 in malignant cells. This preferential binding leads in turn to a serum-independent activation of NHE1. Consequently, activation of NHE1 causes a permanent elevation of

pH_i and thereby results in the abnormal phenotypes of malignant cells, such as high resistance to serum starvation and enhanced cell invasion.

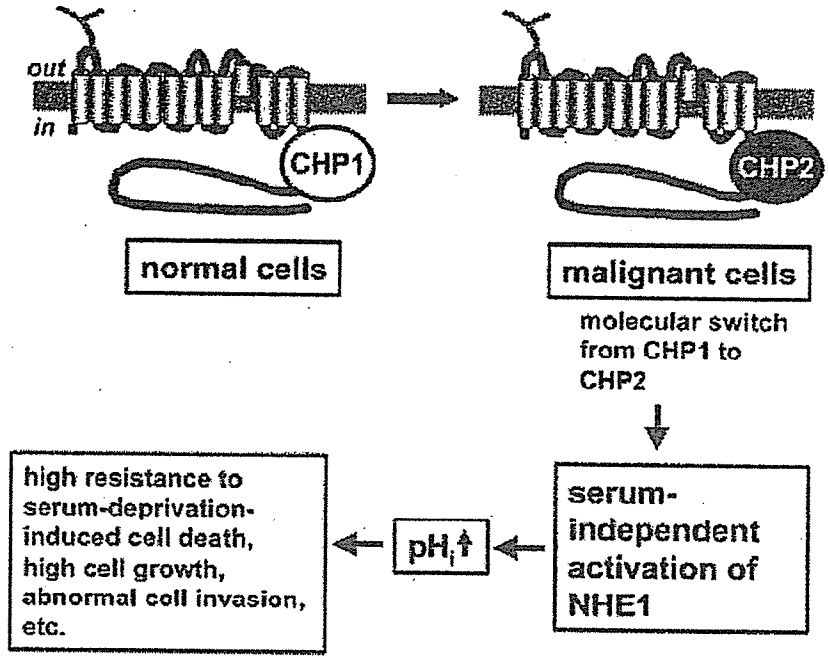


Figure 6. Hypothesis for the role of CHP2 in malignant cells.

4. CONCLUSION

The Na^+/H^+ exchangers are known to interact with various cytosolic factors. In this chapter, we have focused in particular on two important factors, the proton and CHP. We have shown that NHEs basically do not function under physiological conditions unless both factors interact with NHEs. Such observations suggest that these factors are different from other interacting proteins that may be involved in the delicate regulation of exchangers. In this chapter, we also have described the apparent role of CHP2 in maintaining abnormal pH_i in malignantly transformed cells. Future studies, including site-directed mutagenesis, determination of the crystal structure, and physiological experiments using animal models, will be necessary to elucidate more precisely the functions of CHP1 and CHP2 in NHEs and the relationship between these proteins and the "pH-sensor."

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12 放射光および普及型X線源を用いた微小血管造影による再生血管の評価

再生医療分野では，自己幹細胞および遺伝子導入による再生血管治療が臨床応用されつつあり，すでに末梢動脈閉塞症に対する骨髄単核球移植では良好な臨床成績が報告されている．しかし，通常の血管撮影では200 μm 以下の微小血管は描出できず，再生血管治療の効果判定は依然として困難とされている．一方，シンクロトロン放射光はヨードの K 吸収端のエネルギーレベル（33keV）で単色化することにより，周囲組織とのコントラスト効果を最適化し 100 μm 以下の微小血管を描出し，評価することが可能である．また，実際の臨床に導入することを目的として，既存の医療用の高出力 X 線源装置を用いた普及型（病院設置型）微小血管造影装置も開発中で，新たな再生血管治療の評価および診断方法として期待されている．

1．背　景

近年，動脈硬化性の疾病が増加しつつある．特に狭心症や心筋梗塞などの虚血性心疾患，微小循環障害による多発性脳梗塞症，下肢動脈病変を代表する閉塞性動脈硬化症などは豊かな高齢化社会を実現するために，是非とも解決されるべき問題となっている．これらの既存の治療法としては薬物治療やカテーテル治療，また外科的バイパス術などが主に施行されている．しかし，糖尿病合併症例などでは，びまん性微小血管病変が高頻度にみられ，血行再建が困難な難治性症例も少なくなく，既存の治療法では解決できないことも多い．このような症例に対し，新しい治療戦略として血管再生治療による効果が期待されている．循環器領域における再生医療は，血管再生治療と心筋再生療法に大きく分けられており，前者は末梢動脈の閉塞性疾患に対し自家骨髄単核球治療や末梢血幹細胞治療などがすでに臨床導入されており，高率に臨床症状が改善していることが報告されている[1]．後者は今のところ一部を除き研究段階であるが，臨床応用が広く開始されれば，動脈硬化疾患以外にも先天性や後天性心疾患の治療を含めた広範な応用に期待が持てる．

臨床における血管再生治療を行った下肢血管の他覚的評価方法として，一般に血管造影法（DSA）が施行される．しかしながら，臨床症状の改善に比し，血管造影上の有意な改善が見られないことが多い（図Ⅲ-70）．ときに血管数の増加が見られることもあるが，側副血行路の発達（arteriogenesis）と考えられており，再生した血管そのものが造影されているわけではない．微小血管を評価するためには微量の造影剤を検出することができる微小血管造影法が必要になる．微小循環の検出には，高輝度のX線をヨードのK吸収端直上のエネルギーレベルで単色化する方法，X線を平行化し血管端を鮮明に描出する方