Molecular intervention with antisense oligodeoxynucleotides (ODNs) in nephrology

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Manipulation of the expression of a specific gene may shed light on the function of the encoded protein under physiological and pathophysiological conditions as well as may form the basis for fundamental therapy of genetic disorders. Among various strategies for gene modulation, antisense oligodeoxynucleotides (ODNs) complementary to a target mRNA represent a new paradigm for drug discovery [1–3]. Thus, antisense ODNs containing 15–30 residues offer the potential to block the expression of the corresponding gene. Although unmodified phosphodiester ODNs are rapidly degraded by nucleases, chemical modification can increase their stability and potency. The most widely used analogues to date are the phosphorothioate ODNs (S-oligo), in which one of the non-bridging oxygen atoms in the phosphodiester backbone is replaced with a sulphur [4].

Mechanism of inhibition of protein synthesis by antisense ODNs

Various processes on the pathway to protein synthesis are inhibited by antisense ODNs [1]. Thus, such ODNs can induce transcriptional arrest, inhibition of RNA processing, disruption of mRNA structure, or direct inhibition of translation. The formation of a triple-strand structure by double-stranded DNA and antisense ODNs in the promoter region may result in transcriptional inhibition. Triplex-forming ODNs binds to the purine-rich strand of double-stranded DNA via Hoogsteen hydrogen bonds, with base triplet stabilized by two such bonds between the ODNs and its complement.

The activation of RNase H is thought to be a major mechanism of action of antisense ODNs at least in cell-free systems and in Xenopus oocytes. The hybridization of antisense ODNs to complementary mRNA result in RNase H-mediated degradation of the hybridized mRNA, and consequent inhibition of synthesis of the encoded protein. The concentration of RNase H in the nucleus is thought to be higher than that in the cytoplasm because the enzyme participates in DNA replication. Therefore, intranuclear delivery of antisense ODNs is important for effective inhibition of protein synthesis.

For translation arrest, antisense ODNs have been designed to bind to the translational initiation codon. However, targeting the initiation codon does not always result in maximal inhibition of gene expression; antisense ODNs specific for the 3’ non-coding region of mRNAs often provide the greatest inhibition of protein synthesis [5]. However, non-specific effects of ODNs should be taken into consideration. A sequence-specific action of antisense ODNs can be inferred from the selective depletion of the target mRNA and protein. ODNs containing four contiguous guanosine residues occasionally inhibit cell proliferation [6], whereas phosphorothioate ODNs have been shown to activate the SP1 transcription factor [7]. ODNs containing CG motifs with appropriate flanking sequences stimulate the immune system by inducing the production of interferon [8]. A selective decrease in the abundance of the target mRNA and protein should not accompany such non-specific effects of antisense ODNs.

Targeting of ODNs to glomerular cells

Transfer of ODNs to glomerular cells is difficult with conventional methods such as based on cationic liposomes. Instead, gene transfer mediated by haemagglutinating virus of Japan (HVJ)-liposome method appears to be a feasible technique with which we could target glomerular cells [9]. HVJ-liposome method is modified fusogenic liposomes that contain the F glycoprotein on its envelope. HVJ belongs to the paramyxovirus family and can fuse with most cells except lymphocytes via the F glycoprotein. ODNs are encapsulated in the liposome by vortex mixing, and are then fused with HVJ that has been inactivated by ultraviolet irradiation. Transfer of ODNs by this approach results in long-term retention and stability of DNA in the nucleus by an unknown mechanism. Injection of HVJ liposome
containing fluoresceinisothiocyanate (FITC)-labelled ODNs into the rat renal artery was followed 20 min later by the appearance of FITC fluorescence in the nucleus of glomerular cells, mainly in the mesangial area [10]. However, FITC fluorescence substantially decreased and had disappeared after 3 days.

We applied the antisense approach to inhibit the action of transforming growth factor-β (TGF-β) in the anti-Thy1 model of experimental glomerulonephritis [10]. In this model, TGF-β up-regulated in the nphritic glomeruli and is associated with expansion of the extracellular matrix. The amount of TGF-β mRNA and protein are maximal on day 4 and 6, respectively. Introduction of antisense ODNs specific for TGF-β on day 2 inhibited the subsequent accumulation of extracellular matrix as well as the up-regulation of TGF-β mRNA and protein, indicating that TGF-β plays an important role in the disease process. Although this model is self-limiting, treatment with antisense ODNs targeted to the mesangial cells may provide a potential approach to slowing the progression of glomerulonephritis.

Manipulation of tubular gene expression by antisense ODNs

The kidney tubule is composed of different segments in which various proteins, such as ion channels and transporters, confer distinctive functional properties. Antisense ODNs represent a promising approach with which we could shut off the action of an individual protein in an attempt to understand its function. However, specific delivery systems for targeting individual segments in situ remain to be developed. Rappaport et al. [11], examined the organ distribution and cellular localization of 32P-labelled ODNs by autoradiography after intravenous injection in rats. The labelled ODNs were predominantly localized in liver and kidney; in the latter, they were mostly in cells of proximal tubule. These observations suggest that the proximal tubule is an excellent target for proximal tubule-directed antisense therapy. Oberbauer et al. [12] also showed that intravenously injected ODNs accumulated in proximal tubular cells in rats. Electron microscopy revealed that the ODNs were not restricted to the brush border or other individual compartments within proximal tubule cell. Thus, ODNs taken up by proximal tubule cells may not be degraded in the lysosome totally.

Two studies have applied antisense ODNs for the specific inhibition of proximal tubular proteins. Oberbauer et al. intravenously administered antisense ODNs specific for a Na+/Pi cotransporter (3–9 mg/kg body weight) to rats. Transport of phosphate (Pi) was inhibited together with expansion of the Na/Pi cotransporter [13] gene in the proximal tubule. Noiri et al. [14] attempted to prevent acute renal failure in rats subjected to renal ischaemia. They administered antisense ODNs specific for inducible NO synthase (1 mg/kg body weight) to try to inhibit NO production after ischaemic reperfusion injury. The antisense ODNs inhibited NO production and reduced proximal tubular damage. These studies indicate that systemically infused oligonucleotides can exert antisense effects in the renal proximal tubule, which is a major site of their accumulation.

Future perspectives

The first-generation antisense ODNs have reached the stage of clinical trials in humans. More than 10 protocols have been studied and include attempts to inhibit human immunodeficiency virus (HIV) infection and proliferation of cancer cells [4]. However, they do not include attempts to treat renal diseases. Several hurdles must be overcome before the clinical application of antisense ODNs for kidney diseases, including the development of a selective delivery system for targeting individual cell types in kidney, improvement in the efficacy of transfection with ODNs, and establishment of the safety of such an approach. Antisense therapy requires repeated administration of ODNs, given that the antisense effect does not last long. Accordingly, acute renal failure and rapidly progressive glomerulonephritis may be renal diseases that are potential targets for antisense therapy.

References

How does the macula densa sense tubule function?

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Introduction

The macula densa controls glomerular function at least two ways: firstly, it resets tubular glomerular feedback (TGF) so that enhanced delivery to the macula densa leads to a reduction of single-nephron filtration rate (SNGFR) of the respective nephron [1]; secondly, renin secretion is determined by the load to the macula densa, and this may also influence glomerular function. Originally it was thought that renin was the mediator of TGF [1], but this is clearly not the case. TGF is a local readjustment process aimed at the prevention of salt and fluid loss of each individual nephron.

An increased Na\(^+\) and Cl\(^-\) delivery to the macula densa relays either of two messages:

1. one is a ‘local’ message, i.e. that (1) either the SNGFR is too high for the proximal and thick ascending limb (TAL) absorptive capacity of this very nephron, or (2) the absorptive capacity in this nephron is reduced causing an imbalance even at normal SNGFR. In either case a reduction in SNGFR would tend to cure the problem and prevent flooding of the distal nephron with Na\(^+\) and Cl\(^-\) [2].

2. The second is a more ‘general’ message concerning the salt balance. An enhanced delivery of Na\(^+\) and Cl\(^-\) is perceived as volume expansion and renin is suppressed, whereas a reduced delivery is sensed as Na\(^+\) and Cl\(^-\) deprivation and renin is activated. This systemic response, together with other factors controlling renin secretion, such as \(\beta\)-adrenergic innervation of renin-producing cells and a fall in renal perfusion pressure, governs a host of protective mechanisms triggered by the enzyme renin.

In summary, an increased NaCl load to the macula densa reduces SNGFR by TGF and, at the same time reduces renin secretion. A decreased NaCl load has the opposite effect.

The macula densa a ‘chemoreceptor’

There has been much debate as to what parameter is measured by the macula densa cells. Is it the osmolality itself, the concentration of Na\(^+\) of Cl\(^-\) or both [3]? In very elegant microperfusion studies [2,4], Schnermann and colleagues have asked how the TGF is controlled by the macula densa. They have perfused the loop of Henle retrogradely by inserting a perfusion pipette into the earliest distal loop. Hence, they could be certain that the perfusate was altered very little when reaching the macula densa, unlike what is seen with orthograde perfusion through the late proximal tubule. They found that the largest TGF responses were caused by Rb\(^+\) and Na\(^+\) and Cl\(^-\) and Br\(^-\) and the smallest responses were seen with, for example, acetate and choline. This might suggest that Na\(^+\) and Cl\(^-\) are the measured ion species, but it is entirely unclear how the macula densa cells could possibly measure these ions.

Another very intriguing finding is that TGF can be inhibited by loop diuretics such as frusemide [5]. This observation must appear puzzling because loop diuretics undoubtedly enhance the load of NaCl delivered to the distal tubule (and macula densa). Therefore one might have expected that TGF was maximally activated by these substances and that renin secretion is suppressed. The opposite was, in fact, found.

The macula densa cell is just another thick ascending limb cell

Early attempts to analyse the properties of macula densa cells by impalement techniques were not very satisfying [6]. The voltages appeared unreasonably low and were not responsive to frusemide. Schlatter and co-workers [3] took up this issue again and combined \textit{in vitro} perfusion techniques with standard impalement methods. They reported that only very few out of thousands of attempts were technically acceptable. In these few records, however, they obtained reasonable voltages of around \(\approx 60\) mV. Manipulation of the bathing solutions enabled them to characterize these...
cells further. Cl\(^-\) as well as K\(^+\) conductances were found, and most importantly it was reported that the macula densa cells were strongly hyperpolarized by loop diuretics.

The concept emerging from these studies was that the macula densa cell shares in common with the thick ascending limb cell all basic components. The Na\(^+\)2Cl\(^-\) cotransporter is present in the luminal membrane as well as a K\(^+\) conductance. In the basolateral membrane a NPPB-inhibitable Cl\(^-\) conductance and the (Na\(^+\) + K\(^+\))-ATPase are found. Obviously the rate of transepithelial transport is much lower in macula densa cells when compared to thick ascending limb cells [3]. This is not surprising. The macula densa cells take almost no share in the transport work of the thick ascending limb, but they seem to transport only in order to measure the luminal composition.

A few years ago Schlatter [7] has performed comparable studies with the patch clamp rather than impalement techniques. The advantage of the patch clamp studies was that a higher yield of successful recordings could be obtained. With this modified techniques the question as to the mechanism of interaction of diuretics could be examined more closely. It was shown that all loop diuretics examined (torasemide, frusemide, piretanide) hyperpolarized macula densa cells by their specific interaction with the Na\(^+\)2Cl\(^-\) cotransporter, but that hydrochlorothiazide and muzoside had no such effect. These data provide final functional proof for the operation of Na\(^+\)2Cl\(^-\) but not a Na\(^-\)Cl\(^-\) cotransporter in these cells. This concept was subsequently supported by other studies [8].

**Luminal Cl\(^-\) concentration is measured by the macula densa cell**

We can now come back and ask what signal really is sensed. The Na\(^+\)2Cl\(^-\) cotransporter takes up all three ion species. The driving force for this uptake is provided by the (Na\(^+\) + K\(^+\))-ATPase, by keeping cytosolic Na\(^+\) concentration low. The kinetic properties of this transporter, which has been cloned meanwhile [9], have been examined in detail in the thick ascending limb and in the rectal gland of the shark *Squalus acanthius* [10,11]. For both transporters, which share a surprisingly high degree of homology, it was reported that Na\(^+\) and K\(^+\) bind with very high affinity. The affinity for Cl\(^-\) was much lower in the 30–50 mmol/l range. The rate of transport will hence be determined by the luminal Cl\(^-\) concentration, because the Na\(^+\) and K\(^+\)-sites will be occupied completely even at very low concentrations.

The next steps in transduction are not clear at all. The rate of coupled Na\(^+\)2Cl\(^-\) uptake determines the cytosolic Cl\(^-\) concentration and hence the voltage. The higher the luminal Cl\(^-\) concentration, the higher the cytosolic Cl\(^-\) concentration, and the more depolarized the voltage. It has been suggested that the Cl\(^-\) concentration on the basal cell pole will vary correspondingly [12] and that this will reset TGF and control renin secretion. At this stage fairly little is known how renin secretion is controlled in renin-producing cells. It is clear that it is upregulated by elevated cAMP (β-mechnism) and by NO [13] and that it is inhibited by elevated Ca\(^2+\) [14]. The latter mechanism appears to be mediated by an activation of a Cl\(^-\) conductance [15]. Many more details at the cellular level need to be known to complete the puzzle of how macula densa cells ‘talk’ to renin-producing cells. It is clear now, however, that the first and only step is the concentration-dependent luminal uptake of Cl\(^-\).

**Why do loop diuretics paralyse TGF and upregulate renin secretion?**

The above discussion clarifies that loop diuretics ‘cheat’ macula densa cells. By blocking the uptake of Na\(^+\)2Cl\(^-\) specifically they convey the message via the macula densa cells that luminal Cl\(^-\) concentration is low, whilst in fact it is usually isotonic after loop diuretics [10]. The response then must be an interruption of the TGF response and an upregulation of renin secretion. This upregulated renin secretion has nothing to do with volume loss and will prevail even with very careful volume and salt replenishment. This conclusion is clinically important. Whilst other diuretics such as thiazides may also increase renin secretion by a decrease in blood pressure or by volume contraction [2], loop diuretics induce renin secretion directly. The clinician will know from experience that converting-enzyme inhibitors will have an (unexpectedly) strong effect in patients pretreated even with low doses of loop diuretics, simply because the renin–angiotensin–aldosterone system is upregulated by loop diuretics.

**References**

3. Schllatter E, Salomonsson M, Persson AEG, Greger R. Macula densa cells take almost no share in the transport work of the thick ascending limb, but they seem to transport only in order to measure the luminal composition.

Genetics of cardiovascular disease in type 1 (insulin-dependent) diabetes with and without renal involvement

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Introduction
Diabetic nephropathy (DN) is a devastating long-term complication of insulin-dependent diabetes (IDDM) that carries a heavy economic burden and a reduced quality of life. The risk of being affected varies between 10 and 35% with no certain sex difference [1,2]. Chronic hyperglycaemia seems to be necessary but not sufficient for its development. Other susceptibility factors, probably genetically determined, are likely to interact with the diabetic milieu in this process. This is supported by evidence that DN clusters in families with almost a fivefold increased risk among IDDM siblings of probands with DN [3]. A genetic predisposition to nephropathy is further supported by the fact that the risk for overt proteinuria dramatically decreases after the second decade of IDDM, i.e. when genetically susceptible individuals have already been affected.

As the progression of diabetic renal disease is remorseless, primary prevention is necessary. Persistently good metabolic control during the first decades of IDDM probably reduces the risk dramatically [1]. However, as this is difficult to achieve early detection of patients with increased susceptibility is decisive for target intervention. The advantage of genetic markers is that they may be identified independently of age, diabetes duration, or treatment.

The choice of study design of genetics offers different benefits and pitfalls [4]. Association analyses in case-control studies are limited to specific candidate genes based on assumptions of underlying pathogenic mechanisms. On the other hand, due to the multifactorial aetiology of DN the ability to study contributing, and not only necessary, susceptibility loci and their interaction with exposure is of great value. So far, most genetic studies concerning DN have been of this kind. In linkage analyses in family studies no preconceived notions of risk mechanisms are needed. This is an advantage in diseases with a complex mode of inheritance such as DN. On the other hand, when analysing siblings concordant for disease a great number of sibpairs are required. In the analysis of discordant sibpairs, i.e. analysis of diminished allele sharing, fewer numbers are required to obtain similar power. Family studies of DN are currently undertaken in USA and in Europe.

Is family history of cardiovascular disease a risk factor for nephropathy in IDDM?

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in IDDM [5]. Within a few years after the diagnosis of DN around 50% of the patients develop CVD, and well-recognized risk markers of CVD such as hypertension, insulin resistance, hyperlipidaemia, and high fibrinogen levels are all present in clinical nephropathy [5]. However, the aggregation of these cardiovascular risk markers have already been detected at the stage of microalbuminuria, i.e. when renal function is still well preserved [6]. Furthermore, CVD is less common in patients with DN in countries with a low general population rate of CVD [5]. This indicates that CVD may not only be a consequence of the renal disease.

If familial clustering of CVD is evident in IDDM patients with enhanced risk for renal disease, independently of other confounding risk factors, this certainly
supports the hypothesis that these diseases may share genetic traits. In a primary study no such aggregation was detected among parents of patients with DN, probably due to the young parental age [7]. Subsequent data have proposed an approximately threefold excess of CVD and myocardial infarction (MI) in relatives of patients with DN [8]. Furthermore, in a nationwide nested case-control study of IDDM adolescents in Sweden (unpublished) we have found that both hypertension in parents and familial CVD (defined as fatal or non-fatal stroke or MI in parents and grandparents) are significant risk factors for incipient and overt nephropathy, independently of each other. This implicates that different genetic predispositions to hypertension and CVD may be important in determining the risk for nephropathy in IDDM.

Are there any common genetic markers of cardiovascular disease and diabetic nephropathy?

Familial clustering of coronary heart disease (CHD) has long been known. Shared lifestyle may partly, but not completely, explain the excess risk. A genetic predisposition to CHD per se, and not only to its risk factors, is probably a strong contributor, although knowledge of the heredity is still limited, and several genes are believed to be involved. The most extensively studied candidate genes are those involved in the renin–angiotensin system (RAS), due to its altered activity and the benefit of therapeutic inhibition of this system in CHD [9]. The RAS has a profound influence not only on systemic, but also on glomerular circulation and possibly on glomerular structure. Haemodynamic alterations with increased glomerular filtration rate (GFR) and perfusion pressure, modulated by the ACE activity, frequently precedes DN and may play a role in its pathogenesis. Moreover the inhibition of ACE that reduces the conversion of angiotensin I to A II is associated with a reduction of microalbuminuria and a slower rate of decline of GFR in DN [5].

Several molecular variants encoding for components of RAS are identified. Among those the insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin-converting enzyme (ACE) gene on chromosome 17q23 has attracted a great deal of attention as a marker for MI, left ventricular hypertrophy and stroke. The initial report by Cambien et al. [10] found an increased prevalence of DD genotype in otherwise 'low-risk' males after MI. Although their findings have been confirmed by others, the interpretation of the DD genotype as a potent risk marker for MI has also been called in question, mainly because of the possibility of survival bias. In a prospective study, but of a possibly less ethnically homogeneous population, the D allele did not confer any increased risk for MI [11].

The DD-genotype is associated with the highest ACE activity in plasma. As ACE activity is increased in IDDM, and in micro- and macroalbuminuria in particular [12], the I/D polymorphism has been an obvious candidate for research in DN. In a pioneer study Marre et al. [12] found that the II-genotype may protect against DN. Following publications, mostly focusing on the relationship with the D-allele, have been inconclusive [4]. A possible explanation to the inconsistent results may be different selection criteria of patients and controls and variations in genetic backgrounds. By including patients on renal dialysis for instance, the relative risk of specific genetic markers for CVD may be diluted due to survival bias, i.e. susceptible individuals may have died in CVD in the earlier course of DN. Apart from this, the I/D polymorphism may not be a sufficiently informative marker of DN. However, adjacent disease mutations in linkage disequilibrium with the I/D polymorphism, may afford more specific information. An interesting candidate is the 1Dde polymorphism, located on intron 7 and in loose disequilibrium with the I/D polymorphism. It consists of the ‘+’, ‘-‘, and ‘=’ alleles. Results now emerge that the ‘=’ allele is significantly overrepresented in IDDM patients with DN. Interestingly, the ‘=’ allele has been found on haplotypes carrying the D allele, and this haplotype seems to confer a substantial risk increase for DN [4].

A variant (M235T) of the angiotensinogen gene on 1q42–43 has also been implicated in the development of CVD. Mostly, the TT genotype of this polymorphic marker has shown both linkage and association to essential hypertension. Although a large interracial variation exists in the M235T allele distribution, data from different populations have also shown an association between this genotype and CHD [9]. The TT genotype is associated with the highest angiotensinogen levels in plasma that may stimulate overproduction of angiotensin II (A II ), a potent vasoconstrictor and growth factor for vascular smooth cells, which could possibly promote the development of diabetic glomerulopathy. However, in most but not all case-control studies, the M235T polymorphism has not proven a significant candidate gene for DN, also when yielding a sufficiently high power [13,14].

The A/C1166 polymorphism of the angiotensin II type 1 receptor gene (AGT1R) on 3q21–23 has likewise been linked to hypertension. Furthermore, a synergistic effect of the I/D ACE gene and the A/C AGT1R-gene polymorphisms is described on the risk for MI [9]. A genetic variability in the sensitivity to A II may possibly also affect the risk for DN. Of the two A II receptors identified, the AT1 receptor seems to be responsible for the vasopressive actions in the kidney. So far though, no evidence of an independent significant association between the A/C1166 polymorphism and DN has been provided.

However, although single candidate genes may not prove to be sufficiently informative, interactions between polymorphic markers may still modulate the susceptibility of, or protection from, development and/or progression of diabetic renal disease. Preliminary data regarding the impact of polymorphisms in the RAS on the progression of DN during long-standing ACE inhibition suggest that an inter-

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action between ACE/ID + II and AGT1R/AC + CC genotypes afford greatest protection [15]. Besides, when analysing the relative contribution of genetic risk markers on DN it is important to take the interacting (or confounding) effect of exposure, such as metabolic control, diabetes duration, or other known risk factors, into account. Thus a synergistic effect is described between glycaemic control and presence of the C1166 allele of the AGT1R gene [4]. This interesting finding raises the question whether chronic hyperglycaemia is pivotal for the ‘activation’ of genes implicated in the development of DN, as also whether certain polymorphic markers may regulate the renal response to hyperglycaemia. In the only study so far, taking long-term glycaemic control into consideration, the ACE/DD genotype was a marker of DN independent of mean HbA1c during 10 years, whereas long diabetes duration (>20 years) revealed a stronger codominant effect of the D-allele on diabetic renal disease [16].

Final remarks

It may be speculated that the initiation and the rate of progression of DN may not necessarily be influenced by the same genes and/or by the same interactions with other putative risk factors. Prospective studies are necessary to evaluate this. Furthermore, cross-sectional studies using clinical end-points such as end-stage renal failure or microalbuminuria may suffer from survival bias or from misclassification of disease respectively. Thus a complementary approach may be to investigate the influence of polymorphic markers involved in CVD on the patterns of development and progression of the diabetic renal structural lesions underlying the development of clinical nephropathy.

Finally, several putative genetic markers of cardiovascular risk factors (e.g. genes encoding for cation transporters, insulin sensitivity, insulin signalling, and genes involved in lipid metabolism) may also contribute to the pathogenesis of diabetic nephropathy [4], but this is beyond the scope of this editorial.

References

9. Harrap SB. Cardiovascular disease and genetics of the renin–angiotensin system. Heart 1996; 76 [Suppl. 3]: 13–17
Implications of the Systolic Hypertension in Europe (Syst-Eur) Trial for clinical practice

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Introduction

In 1989 the European Working Party on High Blood Pressure in the Elderly started the placebo-controlled double-blind Syst-Eur (Systolic Hypertension in Europe) trial [1]. In view of the remaining uncertainties with regard to the treatment of older subjects with isolated systolic hypertension [2–6], the Syst-Eur trial continued after publication of the SHEP results [7]. The double-blind phase of the trial was stopped on 14 February 1997 at the second of four planned interim analyses. According to the predefined stopping rules, a significant benefit for stroke (Fig. 1), the primary endpoint of the trial, had been reached [8]. This editorial addresses the clinical implications of the Syst-Eur trial.

The Syst-Eur findings

The Syst-Eur patients were at least 60 years old [8]. At three run-in visits 1 month apart their sitting systolic blood pressure on single-blind placebo treatment averaged 160–219 mmHg with a diastolic blood pressure lower than 95 mmHg. After stratification for centre, sex, and previous cardiovascular complications, 4695 patients were randomized. Active treatment consisted of nifedipine (10–40 mg/day) with the possible addition of enalapril (5–20 mg/day) and/or hydrochlorothiazide (12.5–25 mg/day), titrated or combined to reduce the sitting systolic blood pressure by at least 20 mmHg to below 150 mmHg. Matching placebo tablets were employed similarly. Patients withdrawing from double-blind treatment were followed further to facilitate the analysis according to an intention-to-treat principle.

At 2 years (median follow-up) the sitting systolic/diastolic blood pressure fell by 13/2 mmHg in the analyses. According to the predefined stopping rules, a significant benefit for stroke (Fig. 1), the primary endpoint of the trial, had been reached [8]. The between-group blood pressures differences were 10.1/4.5 mmHg (95% CI: 8.8, 11.4/3.9, 5.1 mmHg). Active treatment reduced the total stroke rate from 13.7 to 7.9 events per 1000 patient-years (−42%; P = 0.003) (Fig. 1) [8]. Non-fatal stroke alone decreased by 44% (P = 0.007). In the active treatment group, non-fatal cardiac end-points decreased by 33% (P = 0.03). All fatal and non-fatal cardiac end-points, including sudden death, declined by 26% (P = 0.03). A similar trend was observed for non-fatal heart failure (−36%; P = 0.06), for all cases of heart failure (−29%; P = 0.12) and for fatal and non-fatal myocardial infarction (−30%; P = 0.12). Active treatment reduced all fatal and non-fatal cardiovascular end-points by 31% (P < 0.001). Cardiovascular mortality tended to be less on active treatment (−27%; P = 0.07), but all-cause mortality was not significantly influenced (−14%; P = 0.22).

Clinical implications of the Syst-Eur findings

Isolated systolic hypertension in the elderly

The benefits of antihypertensive treatment in the Syst-Eur study were in relative terms similar to those in six other trials [9–14] in older patients with combined systolic and diastolic hypertension. Overall in these trials, antihypertensive treatment reduced fatal stroke by 33% and cardiovascular mortality by 22% [15]. In a subsequent quantitative review [16], which also included the SHEP trial [7], but not the small Japanese study by Kuramoto [12], these pooled estimates were the same, i.e. 33 and 22%.

Fig. 1. Cumulative rates of fatal and non-fatal strokes in the placebo and active treatment groups of the Syst-Eur trial.

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Whereas the relative benefit of antihypertensive treatment is constant over a wide range of risk, absolute benefit varies widely according to the risk of events experienced by the control group [16]. Among seven intervention trials [7,9–11,13,14,17], the smallest absolute benefit was observed in the Medical Research Council (MRC) trial in young hypertensive patients with diastolic hypertension [17] and the largest in the Swedish Trial in Older Patients with Hypertension (STOP) [11]. Per 1000 patients treated for 5 years, the number of strokes and cardiovascular deaths prevented in the latter two trials [11,17] ranged from 2 to 27 and from 6 to 67 respectively. The absolute reduction of the stroke risk in the Syst-Eur trial was similar to the decrease in the STOP trial, while the absolute benefit in terms of cardiovascular mortality was half-way between the MRC [17] and STOP [11] results.

The prevalence of isolated systolic hypertension rises curvilinearly with age. It averages 8% in sexagenarians and exceeds 25% beyond 80 years [18]. The Syst-Eur results may be readily generalized to these patients. Syst-Eur was the first outcome trial in hypertension which has recruited patients in Eastern as well as Western Europe. Of 8926 patients entered in the registries of screened patients, 52.6% were randomized. The Syst-Eur patients were recruited by population screening, at family practices [19] and at primary and secondary referral centres.

The new classes of antihypertensive drugs

Angiotensin-converting-enzyme inhibitors prolong survival after myocardial infarction [20] and in patients with congestive heart failure [21]. Verapamil and diltiazem reduce morbidity and mortality in post-myocardial infarction patients, provided that left ventricular dysfunction is absent [22,23]. However, the role of the newer classes of antihypertensive drugs in the pharmacological treatment of hypertension remains debated [24,25].

According to the 1993 guidelines in the United States [26], diuretics and β-blockers were the only classes of drugs that have been used in long-term controlled clinical trials and shown to reduce morbidity and mortality. They were therefore recommended as first-choice agents. In contrast, a joint committee of the World Health Organization and the International Society of Hypertension [27,28] was of the opinion that although most clinical trials tested diuretics, centrally acting drugs, vasodilators or β-blockers, often in combination, no evidence was available that the benefits would have been due to any particular class of antihypertensive drugs rather than to the lowering of blood pressure per se. The latter experts recommended that several drugs may be prescribed as first-line treatment of mild sustained hypertension. The Syst-Eur trial now provides evidence that also the newer generations of antihypertensive drugs improve prognosis in a large subset of the hypertensive population.

Recently, several studies [29–33] raised the possibility that the use of calcium-channel blockers would be associated with an increased risk of myocardial infarction [33], higher mortality [32], a greater risk of gastrointestinal haemorrhage [29], and cancer [30,31]. These findings have not always been confirmed. For instance, a nested case-control analysis based on the information taken from the General Practice Research Database in the United Kingdom collected full information on exposure time, but did not find an increased cancer risk in users of calcium-channel blockers or angiotensin-converting-enzyme inhibitors relative to the patients on β-blockers [34]. In general, the previously published observational reports [29–33] left a large margin of uncertainty. In particular, with regard to myocardial infarction, confounding by indication could not be excluded. The first-line antihypertensive agent in the Syst-Eur trial was nitrrendipine, a calcium-channel blocker of the dihydropyridine class [35]. The median duration of exposure in the active treatment group was nearly 2 years. Compared with the placebo group, no changes occurred in non-cardiovascular mortality, the incidence of cancer or the rate of bleeding other than cerebral and retinal haemorrhage.

Conclusions

In summing-up the Syst-Eur trial three conclusions emerge. First, the study has confirmed the SHEP findings [7], in that older subjects with isolated systolic hypertension are suitable candidates for antihypertensive treatment to prevent or postpone cerebrovascular and other cardiovascular complications. Secondly, the newer antihypertensive drug classes, exemplified by the calcium-channel blocker nitrrendipine with the possible addition of enalapril, are equipotent to conventional drugs and may well serve as substitutes for the prevention of cardiovascular complications. Finally, the circumstantial evidence [29–33] for potentially dangerous side-effects of calcium-channel blockers has not been borne out, when put to the more rigorous test of a double-blind placebo-controlled prospective trial with a median follow-up of 2 years.

References

between baseline pressure and benefit from treatment in isolated systolic hypertension. Hypertension 1994; 23: 269–270
22. Yusaf S, Held P, Furberg C. Update of effects of calcium antagonists in myocardial infarction or angina in light of the second Danish Verapamil Infarction Trial (DAVIT-II) and other recent studies. Am J Cardio 1991; 67: 1295–1297


PTH—one can teach an old hormone new tricks

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Action of PTH as a uraemic toxin

Acute exposure to high PTH levels has a stimulatory effect on the function of a number of organ systems and tissues not normally considered to be target organs for PTH [1]. These observations have emerged from studies conducted in human subjects and in laboratory animals with normal kidney function given PTH infusions, and from studies on isolated perfused organs or cells stimulated with PTH. Thus, acute exposure to high concentrations of PTH has been shown to stimulate the secretion of several hormones, e.g. insulin, prolactin, and aldosterone [2], and to increase the antigen-induced response of the immune system, as well as to augment the chronotropy, inotropy, and perfusion of the heart, and to dilate the peripheral vascular bed.
In chronic renal failure (CRF) with permanently elevated PTH levels, the general effect of PTH on non-classical target organs is, on the other hand, inhibitory rather than stimulatory [1]. Thus chronic exposure to excess PTH results in a decrease of the glucose- or potassium-induced insulin secretion, as well as in a reduction of the antigen-induced response of the immune system, and further in an impairment of the energy metabolism of the heart and the skeletal muscles, resulting in decreased cardiac output and dysfunction of the skeletal muscles.

Since these chronic effects of PTH were not seen in parathyroidectomized uremic animals, but could be reproduced in animals with normal kidney function upon chronic infusion of PTH, Massey proposed in 1977 the hypothesis, that PTH might act as a uremic toxin [3].

Other chronic PTH effects include neurobehavioural disturbances and peripheral neuropathy, reduced serum concentrations of testosterone, diminished survival of erythrocytes, reduced tissue and plasma lipolytic activity resulting in hypertriglyceridaemia and accumulation of triglyceride-laden lipoproteins, and pulmonary microcalcifications associated with reduced pulmonary diffusing capacity and elevation of right ventricular pressure and of right ventricular hypertrophy.

**PTH excess induces a state of intracellular calcium toxicity**

PTH enhances the entry of calcium into many different cell types by activating the L-type calcium channels [4]. Thus, acute exposure to PTH leads to a peak increase of the intracellular calcium concentration, that may trigger a biological response, e.g. an increase of the secretion of insulin from the beta islet cells of the pancreas or in an increase of the proliferation of T lymphocytes [5].

Chronic exposure to high PTH levels as in CRF with secondary hyperparathyroidism (sec. HPT), on the contrary, is associated with a sustained elevation of the basal levels of cytosolic calcium [4]. This derangement of the intracellular calcium homeostasis is thought to create basis for some of the abnormalities seen in tissue functions in severe uraemia. The increased cytosolic calcium levels seem to be caused by an increased calcium influx as well as a decreased calcium efflux from the cells. Probably, the continuous PTH-mediated calcium entry leads to an inhibition of the mitochondrial oxidation and of the ATP production. Presumably the fall in ATP contributes to the observed impairment of the activities of the enzymes responsible directly or indirectly for the calcium extrusion out of the cells. Thus the calcium extrusion from the cells is reduced, and the intracellular calcium levels further increased.

Treatment with calcium-channel blockers has been shown to block the PTH-induced entry of calcium, and to prevent the reduced production of ATP and the reduced activity of the enzymes responsible for the \( \text{Ca}^{2+} \) exchange mechanism [4]. During treatment with calcium-channel blockers, the cytosolic calcium levels may therefore remain normal despite elevated circulating PTH levels.

**The effects of excess PTH are receptor mediated**

Convincing evidence indicates that the action of PTH in non-classical target tissues is receptor mediated. Firstly the effects of PTH are associated with an activation of the secondary messenger system, and secondly the actions of PTH on tissue calcium uptake and function can be inhibited by PTH antagonists [4]. Accordingly, messenger RNA for the classical PTH receptor has been demonstrated in almost all body tissues [6,7]. Furthermore, action of PTH via the novel PTH receptors, such as the PTH2 receptor [8] and the carboxy-terminal PTH receptor [9], may contribute to the effects of PTH in organ systems besides the classical targets, kidney and bone. The PTH2 receptor is expressed in a number of non-PTH classical target organs, such as the brain, lung, heart and vasculature, epididymis, and exocrine pancreas [10], whereas the distribution of the receptor specific for the carboxy-terminal region of PTH is presently unknown.

**Cross-reactivity between PTH, PTH-related peptide, and possibly other peptides?**

While PTH is able to evoke biochemical and clinical responses in a number of non-classical target tissues, there is no evidence that PTH is the natural ligand in all of these tissues. Rather the supraphysiological concentrations of PTH needed to elicit a response in these tissues indicate that the effects of PTH are pathophysiological rather than physiological. Most of the non-classical target tissues responding to PTH produce PTH-related peptide (PTHrP), an autocrine/paracrine hormone that binds with the same affinity as PTH to the classical PTH receptors, and which induces a similar activating response of the secondary messengers [11,12]. In fetal life PTHrP is an important hormone for the development and differentiation of the fetus and acts as the most important hormone in calcium homeostasis [12]. It is therefore likely that the natural ligand for the classical PTH/PTHrP receptor expressed in many of the non-classical PTH-responsive tissues in fact might be PTHrP and not PTH, but that the receptors respond to PTH in supraphysiological concentrations. The PTH2 receptor is not activated by PTHrP. Still PTH may not be the only natural ligand for this receptor. Thus in a recent paper Usdin [13] provided evidence for a peptide distinct from PTH, which acted on the PTH2 receptor in the brain with a greater potency than PTH.

**Downregulation of the classical PTH receptors in uraemia**

In the settings of CRF, resistance to the action of PTH is a well-described phenomenon in kidney and bone,
as well as in some of the non-classical tissues, such as the T and B lymphocytes [14]. This may be due to downregulation of the classical PTH receptors, as it has been demonstrated in the kidney, the bones, the liver and in the heart from rats with CRF [15–17]. It has been suggested, that the receptor downregulation may serve as a compensatory mechanism tending to protect tissues from continually high PTH concentrations.

Smogorzewski et al. [17] have proposed that the downregulation of the classical PTH receptors is due to the elevation of cytosolic calcium, which may provide a negative feedback control of messenger RNA for the PTH receptor. This is supported by results demonstrating that both the abnormal cytosolic calcium levels and the abnormal PTH receptor messenger RNA levels could be prevented in CRF rats with sec. HPT by treatment with a calcium-channel blocker. The effect of parathyroidectomy on the PTH receptor expression in uraemia is, on the other hand, not clear from the presently available data [15,18].

The effect of uraemia and sec. HPT on the density of the PTH2 receptors, and on the PTH receptors specific for the carboxy-terminal PTH region is at present unknown.

The toxic effects of excess PTH are preventable, but not always reversible

In the experimental condition, treatment with a calcium-channel blocker can prevent the actions of PTH and can reverse the effects of PTH during short-term PTH elevation [4]. In long-term studies with severely elevated PTH levels, treatment with a calcium-channel blocker may partly normalize the cell or tissue dysfunction. In humans with long-standing uraemia and sec. HPT the effect of treatment with a calcium-channel blocker on the uraemic symptoms seems to be of limited value. This may be due to irreversible changes of cellular and tissue functions due to long-term exposure to high PTH levels. Thus, an enhancement of the interstitial fibrosis by excess PTH, as has been described in the heart of the uraemic rat [19], may play an important role, and possibly other complications may supervene, e.g. acceleration of atherosclerosis. The present knowledge of the long-term effects of excess PTH on the non-PTH classical target organs is sparse.

In conclusion, the many actions of excess PTH on target tissues other than the classical targets, bone and kidney, might be mediated via binding of PTH to the common PTH/PTHrP receptor. This receptor is distributed ubiquitously and is of physiological significance in fetal life, responding to PTHrP. In the adult mammalian organism, however, the physiological importance of PTHrP and its receptor is as yet not clearly characterized—an autocrine/paracrine factor? Therefore, by the demonstration of the common receptor for PTH and PTHrP the concept of PTH as a uraemic toxin has gained further support.

References

17. Smogorzewski M, Tian J, Massry SG. Down-regulation of PTH/PTHrP receptor of heart in CRF: Role of (Ca$^{2+}$), Kidney Int 1995; 47: 1182–1186
Malnutrition is bad, but how can one detect malnutrition?

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Introduction

Renewed interest in nutrition in patients on haemodialysis and peritoneal dialysis was stimulated by the observation of a tight correlation of plasma albumin concentrations and morbidity and mortality [1]. Low plasma albumin was regarded as a reflection of undernutrition, and consequently identification of factors affecting nutritional state and interventions to alleviate malnutrition have become major fields in clinical research in the haemodialysis population in the last decade.

Unfortunately we had to learn that plasma albumin concentration does not necessarily reflect nutritional state. In fact albumin is a negative acute-phase reactant, and this implies that any intercurrent acute disease process (as indicated by a rise in C-reactive protein) will depress hepatic albumin synthesis and lower plasma albumin concentrations [2]. Albumin concentration, thus, is not only affected by the adequacy of nutrient availability and/or by nutritional state.

In this issue of Nephrology Dialysis Transplantation, Marcen and co-workers report a high frequency of malnutrition in chronic haemodialysis patients when evaluated by anthropometric measurements but they could not identify any correlation of these nutritional parameters and mortality [3]. Prognosis, however, was rather related to age and comorbid conditions, such as cardiovascular and neurological disease (lymphocyte count was the only maker that might be related to nutrition).

These findings might cast some doubt on the dogma of an association of nutritional state and morbidity and prognosis. Obviously, a demonstration of a correlation of nutritional markers and outcome does not imply a causal relationship. If a disease process negatively affects both survival and nutritional state, it does not necessarily mean that this effect is mediated via malnutrition. But neither is the opposite conclusion justified or correct, i.e. that malnutrition has nothing to do with morbidity/mortality if proposed nutritional parameters do not correlate with outcome. Furthermore, it might well be the case (as with serum albumin) that we do not have the proper instrumentarium to evaluate malnutrition and do not have at hand reliable indices to quantify proposed deficiencies.

Certainly we should not throw out the baby with the bathwater, and there are numerous experimental and clinical studies which show that nutrient deficiencies and/or malnutrition affect several biological functions such as wound healing and immunocompetence, and that these functions are essential for improving morbidity and mortality in various clinical situations. There are much fewer investigations proving the converse, i.e. that a defined nutritional repletion in fact improves morbidity/mortality [5].

There is no single magic marker of malnutrition

But what then is malnutrition; how should we measure the nutritional state? If we look at other plasma proteins, which have frequently been used as parameters of nutritional state, we will again be disappointed. Transferrin concentration is affected by the iron status (which is altered in haemodialysis patients), production of retinol-binding protein is upregulated in uraemia, and renal catabolism of prealbumin (as of other peptides and proteins) is retarded with decreased metabolic function of the kidney. Thus plasma concentrations of these proteins, again, are poor indicators of nutritional state.

The most popular methods of assessment of nutritional state in non-renal patients are anthropometric measurements. The altered distribution between fat and protein in renal failure patients (with a low triceps skinfold thickness in the majority of subjects), and the broad range of values also in the healthy ‘reference’ population limits the value of these measurements, at least when evaluated at a single time point only. As in the healthy population there is no reason to believe that a fragile body shape necessarily implies undernutrition and/or an increase in morbidity. In fact groups other than Marcen et al. have reported on a poor correlation of anthropometric measurements with other ‘nutritional parameters’ in both haemodialysis and peritoneal dialysis patients [4,5].

The same is true for bioimpedance measurements,

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which have the further limitation that the method is poorly standardized in subjects with rapid fluid shifts and has not been properly validated in haemodialysis/ peritoneal dialysis patients [6].

Numerous other parameters are used to define nutritional state which cannot be discussed in detail here. It must be stressed that many of these multiple markers, in fact, are influenced by nutritional factors and/or reflect body composition. The problem is that they are also affected by various other conditions not related to nutrition, and thus their specificity is low.

In discussing markers of malnutrition, we must not confuse those parameters that (might) reflect nutritional state, and those indices for short-term evaluation of adequacy of nutrient intake. Evaluation of protein catabolic rate by urea modelling (which, in addition, obviously depends on many assumptions such as constant protein intake, absence of intercurrent catabolic factors, etc.) is one of those, and plasma prealbumin, a plasma protein with a short half life is another. Synthesis and also plasma concentration of prealbumin is affected by nutrient availability and the same is true for plasma/intracellular amino-acid concentrations.

One-point measurements of nutritional parameters are worthless

With the broad interindividual range of measurements and the fact that most ‘nutritional indices’ are not determined or affected only by nutritional factors, one has to realize that a momentum view with single measurements of any of these parameters is actually meaningless. What is mandatory is the proper observation over time of the patient and of his physical state and of the evolution of various nutritional indices. Such a practice will also render anthropometric measurements very useful parameters of nutritional state. Even a simple marker such as body-weight will become valuable.

Functional parameters

Malnutrition, by definition, only can become relevant if the functional state of the organism is compromised: if physiological functions, such as immunocompetence, wound healing, muscle strength, etc. are impaired. Therefore functional markers may be much more valuable for evaluation of the adequacy of nutritional status (and also for evaluation of the efficiency of nutritional interventions) than laboratory indices.

One of those parameters is immunocompetence, which is not only reflected by absolute lymphocyte count, but skin-test reactivity (again to be monitored over time). Other markers are evaluation of muscle strength, and physical performance. Last but not least, the functional state of the organism is perfectly reflected by general wellbeing of the patient. Several scoring systems have been defined in various patient populations, integrating the subjective perception of the patient. The Karnovsky index, mostly used in oncological patients might be too rough a parameter, but there are several other scoring systems, such as the ‘subjective global assessment score’ (SGA) which can be evaluated also in haemodialysis patients [8].

The importance of functional parameters is also underlined by the fact that ‘plain clinical judgment’ is still a reliable method to detect undernutrition and the consequences thereof [9]. Obviously, this subjective evaluation does not allow a quantification, and comparable, objective methods are needed.

Composite nutritional scores

With the low specificity and sensitivity of any of the anthropometric and biochemical markers of malnutrition it becomes clear that no single factor properly reflects nutritional state, that there is no magic index of malnutrition. Malnutrition can only be described by a combination of various factors integrating biochemical makers, anthropometric measurements, and evaluation of functional parameters and subjective wellbeing of the patient.

Several groups have started to define such composite nutritional scores also for patients on haemodialysis and/or peritoneal dialysis [10]. But what is lacking—what is urgently needed—is an internationally accepted standard definition of malnutrition in these patient populations: what elements such a composite nutritional score should integrate. The nephrological community should come to a consensus on how to define malnutrition, how to evaluate impairment of the nutritional state in patients on haemodialysis and peritoneal dialysis and, last but not least, when nutritional interventions are indicated and how the efficiency of any intervention should be assessed.

Nutrition is more than calories and nitrogen

Finally, malnutrition is often viewed as inadequate supply of nitrogen and/or calories only (‘protein–energy malnutrition’). There is much more to nutrition than ‘nitrogen and calories’ and we begin to learn that numerous nutrients, such as vitamins, trace elements, carnitine, specific amino acids, etc. may exert important physiological functions [11]. Immunonutrition, antioxidative potential, antiatherosclerotic factors all are labels reflecting a change in the understanding of nutrition. Deficiencies in these factors can induce much more subtle alterations of the nutritional state than can be detected by changes in body-weight, but which might induce important functional alterations. So our perception of nutrition must shift from a quantitative point of view to a qualitative understanding of the pathophysiological relevance of individual nutrients.
References


