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ARTICLE

Molecular characterisation of the defective α1-antitrypsin alleles PI Mwürzburg (*Pro369Ser*), Mheerlen (*Pro369Leu*), and Q0lisbon (*Thr68Ile*)

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Deficiency of the serine proteinase inhibitor (serpin) α1-antitrypsin (α1AT) is the most common autosomal recessive genetic disorder in Northern Europe. α1AT is the physiological regulator of the proteolytic enzyme neutrophil elastase and severe deficiency states are associated with an increased risk of developing chronic obstructive pulmonary disease (COPD) as a consequence of chronic proteolytic damage to the lungs. Among the known mutations of the *α1AT* gene causing severe α1AT deficiency and COPD a few alleles are also associated with liver disease. When expressed in cell cultures, all these particular alleles cause intracellular α1AT accumulation which appears to be a prerequisite for the development of hepatic injury. Liver disease is seen in only a small fraction of all patients carrying such alleles, however. The reason for this is not completely clear, but there is evidence that PI ZZ individuals 'susceptible' to liver disease carry an additional defect affecting protein degradation in the endoplasmic reticulum (ER). We characterise a newly identified defective α1AT allele PI Mwürzburg (Pro369 [*C*CC] to Ser [T*C*C]) associated with a complete intracellular transport block in cell cultures *in vitro*. The allele PI Mheerlen, a previously described different amino acid substitution in the same position as PI Mwürzburg (Pro369 [C*C*C] to Leu [C*T*C]) is shown to cause complete retention of the mutant α1AT in the ER, too, whereas in the recently described mutant allele PI Q0lisbon (Thr68 [A*C*C] to Ile [A*T*C]) a significantly reduced α1AT secretion from the cells was observed. Adenovirus-mediated recombinant expression of mutant Mwürzburg and Mheerlen, and of wild-type α1AT in mouse liver *in vivo* showed that the mutant human proteins were not secreted into the mouse plasma, in contrast with human wild-type α1AT which circulated at high concentrations over several weeks. In summary, all transportation deficient α1ATs analysed have the potential to cause lung disease in the homozygous state or in heterozygous carriers of another deficiency allele, and they may also cause liver disease in certain patients. The mutant PI Mwürzburg and

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Mheerlen α1ATs are completely retained within synthesising cells, and the molecular defect of transportation in these two alleles may be similar to that in the common PI Z allele. The molecular defect in the PI Q0lisbon allele (Thr68Ile) shows similarity with the immediately neighbouring Mmineral springs mutation (Gly67Glu).

Keywords: α1-antitrypsin deficiency; intracellular protein transport deficiency; pathogenetic modelling of human disease; adenovirus-mediated gene transfer

Introduction

α1-antitrypsin (α1AT) deficiency is the most common autosomal recessive genetic disorder among Caucasians of Northern European descent.[1] α1AT is a 52-kD glycoprotein which is mainly synthesised in the liver and secreted into the bloodstream, from where it diffuses into most organs including the alveolar space of the lung.[2,3] In blood and lung α1AT serves as the major physiological inhibitor of the serine protease neutrophil elastase (NEL) which destroys elastin, a macromolecule that provides elastic recoil to the lung.[4,5] Severe deficiency states are associated with an increased risk of developing chronic obstructive pulmonary disease (COPD) due to proteolytic damage to the lungs mediated by elastase.[6,7] α1AT deficiency in combination with cigarette smoking is associated with a significantly reduced life expectancy.[8,9] In addition to this pulmonary disease which is usually not clinically manifest until the third decade of life a minority of the α1AT-deficient individuals develop symptoms and signs of liver disease during childhood, ranging from transient cholostatic icterus to complete liver cirrhosis.[10–12] A wide spectrum of mutations in the *α1AT* gene has been described[7,13] and many of them are associated with severe α1AT deficiency and COPD. Only a few of the deficiency alleles are also associated with liver disease, among them the most common defective α1AT allele PI Z[14,15] which has an allele frequency of 2% in Northern Europe. Other rare alleles associated with liver dysfunction are PI Mmalton[16,17] and Mduarte.[18] All alleles associated with liver disease show intracellular α1AT accumulation. This appears to be a prerequisite for the development of liver injury in mutation carriers which is not observed in alleles with missing protein synthesis such as PI Q0bellingham[19] or Q0granite falls.[20] Interestingly, only a minor fraction of the patients with intracellular α1AT accumulation actually develops liver disease. There is evidence that in addition to the PI ZZ genotype a defect in a common endoplasmic reticulum (ER) protein degradation pathway is required to trigger the hepatopathy,[21–23] and that patients without that defect are protected against liver damage by the mutant α1AT. 11% of children with the PI ZZ phenotype develop cholostatic icterus and 2–3% of them die of liver cirrhosis before the age of 8 years.[20] For the PI Z allele the mechanism causing intracellular α1AT accumulation has been elucidated in molecular detail.[24]

We characterise a new defective allele PI Mwürzburg (Pro369Ser) associated with intracellular α1AT accumulation. In addition, we show that the previously identified allele Mheerlen[16] which carries a different amino acid substitution (Pro369Leu) in the same position as Mwürzburg, also causes complete retention of the mutant protein in the ER. In contrast to the complete transport block associated with these two mutations the deficiency allele PI Q0lisbon (Thr68Ile)[25] is shown to be associated with reduced secretion of α1AT from the cell. Adenovirus-mediated gene transfer[26–30] was used to express the deficiency alleles Mwürzburg and Mheerlen and wild-type α1AT in mouse liver and to study their respective expression kinetics *in vivo*. All transportation deficient α1ATs analysed have the potential to cause lung injury in the homozygous state or in heterozygous carriers of another deficiency allele, and may also cause liver disease in certain patients.

Materials and Methods

Identification of the α1-Antitrypsin Deficiency Alleles PI Mwürzburg, Mheerlen, and Q0lisbon

We observed the deficiency allele PI Mheerlen described by Hofker *et al*[16] in heterozygous form, together with the non-expressing PI Q0granite falls allele,[20] in one of our patients with severe COPD and an extremely low α1AT serum level of 0.7 mM (3.7 mg/dl).[31] The 42-year-old patient had smoked until the age of 36. He had a markedly enlarged intrathoracic gas volume and large emphysematous bullae in both lungs which were rapidly progressive despite α1AT substitution and antiobstructive therapy. He died at the age of 42 of respiratory failure. He had no evidence of liver disease. His family is shown in Figure 1A. We have recently identified the allele PI Q0lisbon in a family of Portugese origin.[25] The index case was a 12-year-old boy suffering from bronchial asthma. He and his mother had reduced α1AT serum levels around

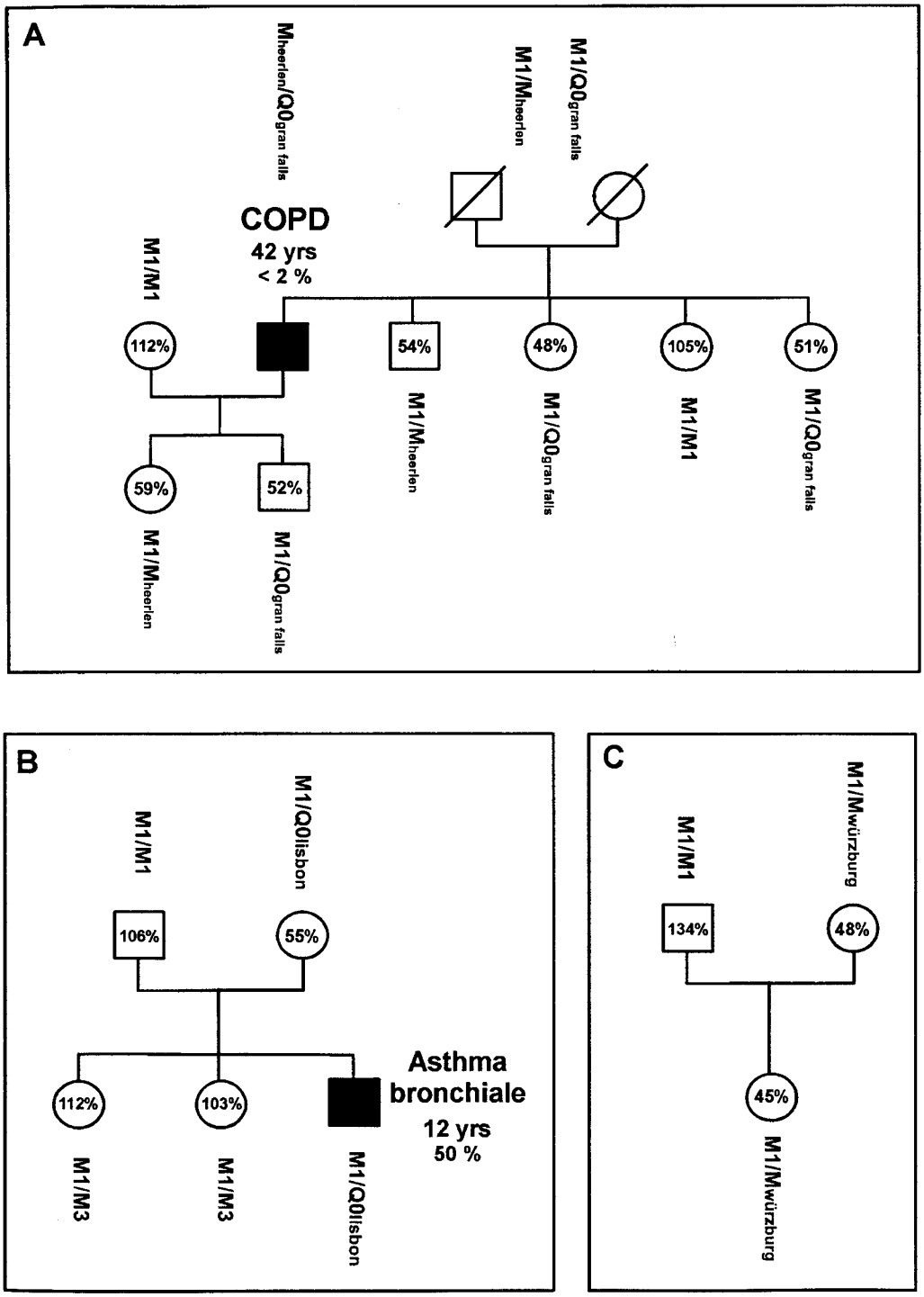

Figure 1 *Familial inheritance of the defective alleles PI Mwürzburg, Q0lisbon, and Mheerlen. Panel A shows the family of a patient (■) with severe COPD who inherited the deficiency allele Mheerlen and the non-expressing Q0granite falls. None of his relatives had lung disease (□: males, ○: females) consistent with their α1AT genotypes. The patient died at the age of 42 y of lung failure. Panel B shows the family of a 12-year-old patient with bronchial asthma heterozygous for the defective Q0lisbon and the normal M allele. Since he had α1AT serum levels above the protective threshold, his asthma is probably not related to the gene defect. Panel C shows the family of a healthy proband with subnormal α1AT serum level who is heterozygous for the new Mwürzburg allele.*

18 mM (94 mg/dl) corresponding to about half the normal value (Figure 1B) and were diagnosed as Q0lisbon heterozygotes by sequencing of their *α1AT* genes.[25] The new deficiency allele PI Mwürzburg (named after the birthplace of the index case) was identified by sequencing of the α1AT genes as described[25] in a heterozygous individual with serum α1AT deficiency but no evidence of lung or liver disease.

Quantification and Isoelectric Focusing of α1-Antitrypsin

Serum levels of α1AT were measured by radial immunodiffusion assay using NOR-partigen™ plates, or LC-plates™ (Behring, Marburg, Germany) for patients with very low α1AT levels. As the normal range of α1AT serum levels we assume 20–53 mM (104–276 mg/dl) and as the critical minimum level necessary for protection of the lungs against proteolytic damage 11 mM (57 mg/dl). The proteinase inhibitor (PI) phenotype of α1AT was determined using hybrid isoelectric focusing of the protein in an ultranarrow pH gradient as described.[32]

Sequence Analysis of the Defective α1-Antitrypsin Genes

All α1AT coding exons including 150 bp 5' to exon Ic and the exon–intron junctions were amplified by PCR using specific primers and genomic DNA of the probands as described.[33] Sequencing was performed by using an automated non-radioactive protocol.[34]

PCR Mutagenesis of Wild-type α1-Antitrypsin-cDNA

After the identification of the point mutations causing the deficient α1AT variants Mwürzburg (Pro369Ser) and Q0lisbon (Thr68Ile), these mutations were introduced into the wild-type cDNA of α1-antitrypsin using the PCR overlap mutagenesis method essentially as described by Ho *et al*.[35] In contrast to the original protocol, we have used the proofreading thermostable DNA Pfu polymerase (Stratagene, Heidelberg, Germany) instead of Taq polymerase. To generate the Mwürzburg mutation the following PCR primer pairs were used: 5'-GTCAAGTTCAACAAA**T**CCTT-3' with the primer 5'-CGGGATCCGAATTCAGT TATTTTTGGGTGG-3' (3'-α1AT), and 5'-AAG G**A**TTTGTTGAACTTGAC-3' with the primer 5'-GGGGTACCGAATTCGACAATGCCGTCTTCT-3' (5'-α1AT). For Mheerlen the respective primer pairs were: 5'-GTCAAGTTCAACAAAC**T**CTT-3' with 3'-α1AT, and 5'-AAG**A**GTTTGTTGAACTTGAC-3' with 5'-α1AT. For an additional mutation described recently (Q0lisbon)[25] the pairs were 5'-CTGGGGA**T**CAAGGCTGACACT-3' with 3'-α1AT, and 5'-AGTGTCAGCCTTG**A**TCCCCAG-3' with 5'-α1AT. For each mutation the two partially overlapping primary PCR products were used as templates in a secondary (overlap mutagenesis) PCR with the primers 5'-α1AT and 3'-α1AT. The parts of these primers which are homologous to the respective ends of the α1AT-cDNA are underlined above. The primers 5'-α1AT and 3'-α1AT have extensions with Eco RI restriction sites which were used for cloning of the overlap PCR product into the eukaryotic expression vector pZS2. The cloned mutagenised PCR products were sequenced completely to verify the efficacy of the mutagenesis procedure.

Expression of the Defective α1-Antitrypsin Alleles in Human Cell Cultures

Synthesis, intracellular transport, and secretion of the deficiency alleles were studied by using metabolic labelling and immunoprecipitation in pulse-chase experiments as described.[28] Expression plasmids for the mutagenised α1AT-cDNAs were transfected into human 293 cells by lipofection using Lipofectamine™ (Gibco, Eggenstein, Germany). Immunoprecipitation included a preclearing step, then polyclonal goat anti-human α1-antitrypsin antiserum (Dako, Hamburg, Germany). Endo–b–N-acetylglycosaminidase H (Endo H) (Boehringer, Glastrup, Denmark) digestion of metabolically labelled α1AT followed the protocol of the manufacturer.

Development of Replication-Deficient Adenoviral Vectors for Defective α1-Antitrypsin Alleles

Recombinant adenoviral vectors for wild-type α1AT and the defective α1AT mutants PI Mwürzburg and Mheerlen were developed as described.[28–30] PCR screening for recombinant viruses was done using the primer pairs 5'-GAATTT-CAACCTCACGGAGAT-3' (α1AT-code) with 5'-CAGCTCCTCGGTCACAT-CCA-3' (Ad5-anti), and 5'-ATCGTGGGTGAGTTCATTTTC-3' (α1AT-anti) with 5'-TGATAATGAGGGG-GTGGAGTTTGT-3' (Ad5-code).

Expression of Defective α1-Antitrypsin Genes in Mouse Liver in vivo

The adenoviral vectors expressing wild-type α1AT and the alleles Mwürzburg and Mheerlen, respectively, were tested for functionality on human umbilical vein endothelial cell (HUVEC) cultures as described,[28–30] in pulse-chain experiments essentially as outlined above. The vectors in a 'standard' dose of 2×10^{10} particles per animal were then injected in four groups of adult C57BI/6 mice (no vector, wild-type, Mwürzburg, Mheerlen), with three animals in each group. To analyse vector dose-dependence of transgene expression, additional mice were injected with 'high' vector doses of 1×10^{11} particles per animal. The mice were 6 to 10 weeks old when injected with the vectors. At different intervals up to three weeks after injection, blood samples were taken by tail vein bleeding, and plasma was assayed by using a highly sensitive and strictly species-specific radial immunodiffusion (RID) assay (LC Plates™, Behring), which does not recognise mouse α1AT, but selectively detects human α1AT down to a concentration of 0.8 mM (4 mg/dl). In an independent experiment, the animals were killed at different times after gene transfer and the livers were taken for examination of transgene expression by northern analysis, and for morphological and histological examination.

Northern Blot Analysis of a1-Antitrypsin Transgene Expression

Radiolabelled single-stranded (ss) DNA probes were synthesised by PCR using human α1AT-specific primers AAT-code-2 (5'-GAATTTCAACCTCACGGAGAT-3') (coding) and AAT-anti-2 (5'-ATCGTGGGTGAGTTCATTTTC-3') (anticoding), and cloned human α1AT-cDNA as template. In a parallel PCR reaction, rat cytoplasmatic β-actin fragments were amplified as described.[36] The specific PCR probe fragments were excised from agarose gels and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). 25-ng portions of these PCR fragments were labelled in a

35 cycle PCR-like reaction: 2.5 µl PCR buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer), 1 µM antisense primer, 20 µM each dATP, dTTP and dGTP, 1.25 U Taq DNA Polymerase and 0.4 µM [^{32}P]dCTP (Amersham) in a 25 µl reaction. Radiolabelled ss probes were separated from non-incorporated dNTPs using Sephadex-G-50 columns (Pharmacia, Freiburg, Germany). Blots were hybridised with the a1AT probe, then stripped and reprobed with the β-actin probe. The radioactivity of the signals was quantified using a GS-250 Molecular Imager™ (BioRad, Munich, Germany).

The α1AT mRNA levels in Fig. 5 are calculated in relative units as the ratio of α1AT signal/β-actin signal strength.

Liver Histology and Immunohistology after Adenovirus-mediated α1AT Gene Transfer

Liver specimens taken one to three weeks after gene transfer were fixed in 10% phosphate-buffered formalin (Sigma, Deisenhofen, Germany) and embedded in paraffin. Four micrometer thick sections were cut and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) with and without diastase digestion. Alternate sections were dewaxed and incubated with a rabbit-anti-human-α1AT antibody (Dako, Glostrup, Denmark) at a dilution of 1:4000. Visualisation of bound antibody was achieved by using the alkaline anti-alkaline phosphatase method[37] using new fuchsin as chromogen. A human liver section from a PI Z individual was used as a positive control with each series of sections. Incubation of the sections with the secondary antibody without primary antibody was applied as negative control.

Results

Evaluation of the Index Cases for the α1-Antitrypsin Alleles PI Mwürzburg and PI Mheerlen

We have observed the PI Mheerlen allele[16] in a COPD patient in combination with the non-expressing allele PI Q0granite falls.[31] The patient was the only affected member of a three-generation family and suffered from rapidly progressive COPD causing his death at the age of 42 years. The very severe course of the disease was obviously triggered by the patient's smoking habit of 20 pack-years, but its genetic basis was the extremely low α1AT serum level of 3.7 mg/dl (0.7 mM) which is only 6% of the minimal protective threshold. In fact, one of his α1AT genes is non-expressing and the other is a severe deficiency allele, but only the combination of both has pathogenetic potential, as shown by the clinically inapparent inheritance of either in the other family members (Figure 1A). In a second family the deficiency allele PI Q0lisbon was observed in heterozygous form in two cases and one of them had bronchial asthma. Both had clearly subnormal α1AT serum concentrations around 18 mM (94 mg/dl), but since in the index case the α1AT level was still well above the protective threshold of 11 mM (57 mg/dl) and his mother had no symptoms or signs of pulmonary disease, his asthma is probably unrelated to his α1AT gene defect (Figure 1B). In the third family there was no evidence of lung or liver disease and the deficiency allele only caused asymptomatic α1AT serum deficiency (Figure 1C).

Expression of the Defective α1AT-Alleles PI Mwürzburg and Mheerlen in Cell Culture

Pulse-chase experiments performed to study synthesis and secretion kinetics of the three defective α1AT alleles PI Mwürzburg, Mheerlen, and Q0lisbon are shown in Figure 2. Three days after lipofection of the respective mutagenised α1AT-cDNAs into 293 cells, the transfected monolayers were labelled metabolically. Immunoprecipitation analysis of intracellular and secreted α1AT newly synthesised during the pulse phase then showed similar amounts of intracellular α1AT for all three mutant proteins and for the normal PI M1 allele (Figure 2, lane 2 in panels A–D). However, at the end of the pulse phase a considerable amount of labelled PI M1 protein was already secreted from the cells (Figure 2, lane 1 in panel D). In contrast, no PI Mwürzburg or Mheerlen protein was detected in the medium at this time (Figure 2, lane 1 in panels A/B). PI M1 protein continued to be secreted from the cells during the following 4 hours of chase (Figure 2, lanes 3/4 in panel D), whereas no PI Mwürzburg or Mheerlen protein appeared in the medium during this time (Figure 2, lanes 3/4 in panels A/B), indicating a complete intracellular transport block for these two mutants. Treatment of the intracellulary retained PI Mwürzburg and Mheerlen proteins with endoglycosidase H showed that both mutant proteins remain Endo H-sensitive (Figure 3) indicating that they do not pass through the ER. Similar experiments using adenovirus-mediated gene transfer to HUVEC cultures gave higher transgene expression levels, but otherwise similar results.

Expression of the Defective α1AT-Alleles PI Mwürzburg and Mheerlen in vivo

Intravenous injection of three groups of mice with recombinant adenoviral vectors expressing either wild-type α1-antitrypsin or the Mwürzburg or Mheerlen alleles, respectively, resulted in grossly different concentrations of recombinant human α1AT in the mouse plasma. Human wild-type α1AT was secreted into the plasma, where it circulated at high concentrations during a period of 3 weeks (Figure 4). In contrast, no human Mwürzburg or Mheerlen allele was detectable

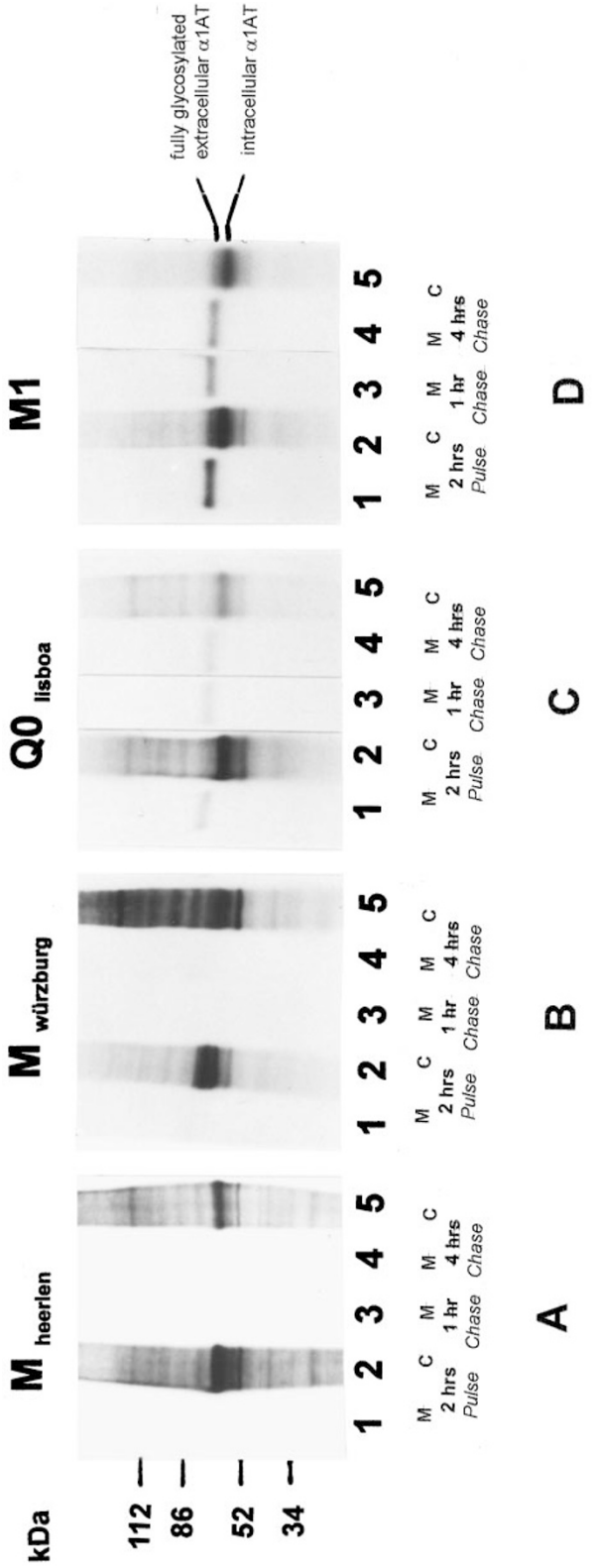

Figure 2 *Characterisation of defective α1AT alleles* in vitro. *Pulse-chase experiments were performed on day 3 after lipofection of 293 cells with the cDNAs encoding the defective α1AT alleles Mheerlen (panel A), Mwürzburg (B), Q0lisbon (C), or the normal M1 allele (D), respectively. Panel D: Normal M1 protein is synthesised during the 2 h pulse phase (lane 2) and a major fraction of the recombinant protein is secreted into the medium during this time in glycosylated form (lane 1). The positions of the intracellular (**C**) α1AT and the larger fully glycosylated a1AT in the medium (**M**) are indicated. After 1 and 4 h chase (lanes 3, 4) more intracellular α1AT labelled during the pulse phase is secreted, and nearly all α1AT is eliminated from the cells after 4 h (lane 5). Panel A: Mheerlen protein of the same apparent molecular mass as the M1 protein is synthesised during the pulse phase (lane 2), but no recombinant α1AT is secreted into the medium during this time (lane 1) or after 1 and 4 h chase (lanes 3, 4). A major fraction of the initially synthesised Mheerlen protein is still detectable in the cells after 4 h (lane 5). Panel B: Similar results as for Mheerlen were obtained for the Mwürzburg protein, which appears to be synthesised regularly, but also shows a complete block of secretion. Panel C: The protein product encoded by the cDNA carrying the Q0lisbon mutation has the same apparent molecular mass as M1 α1AT. In contrast to Mwürzburg and Mheerlen, a fraction of the Q0lisbon protein synthesised in the cell (lane 2) is secreted during the pulse phase (lane 1), but this fraction is much smaller than for the normal M1 allele (panel D, lane 1) and remains very small after a 4 h chase (lane 4). Whereas expression of normal α1AT yields a single band of intracellular protein (panel D, lane 5), the transportation defective alleles Mwürzburg and Mheerlen (and to a lesser degree Q0lisbon) yield high molecular weight bands (panels A–C, lanes 5) possibly representing aggregates of mutant protein.*

in the plasma (< 0.8 mM or 4 mg/dl), although in cell cultures the respective vectors produced intracellular levels of α1AT similar to those of the wild-type vector. Northern blot analysis of total RNA isolated from liver of mice injected with the respective vectors documented regular *in vivo* expression of the transgenes, but showed a gross discrepancy between the steady-state levels of α1AT-mRNA expressed in the liver (Figure 5) and the levels of recombinant human α1AT circulating in the mouse plasma (Figure 4). When compared with the control group receiving luciferase control vector, wild-type α1AT vector enhanced α1AT-mRNA levels up to 3.6-fold on day 4 and to 8.8-fold on day 12 after gene transfer. In comparison, α1AT-mRNA levels were enhanced up to 3.2-fold on day 4 and to 3.6-fold on day 12 after injection of the Mwürzburg vector. Similar results were obtained for the Mheerlen vector. The intracellular transport block observed for the mutant allele *in vitro* was thus reproduced *in vivo*, simulating *one* aspect of the situation in patients carrying transportation-defective α1AT alleles. Liver histology did not reveal significant differences between mice injected with wild-type, Mwürzburg or Mheerlen vector. Liver architecture was normal in all samples, and no PAS-

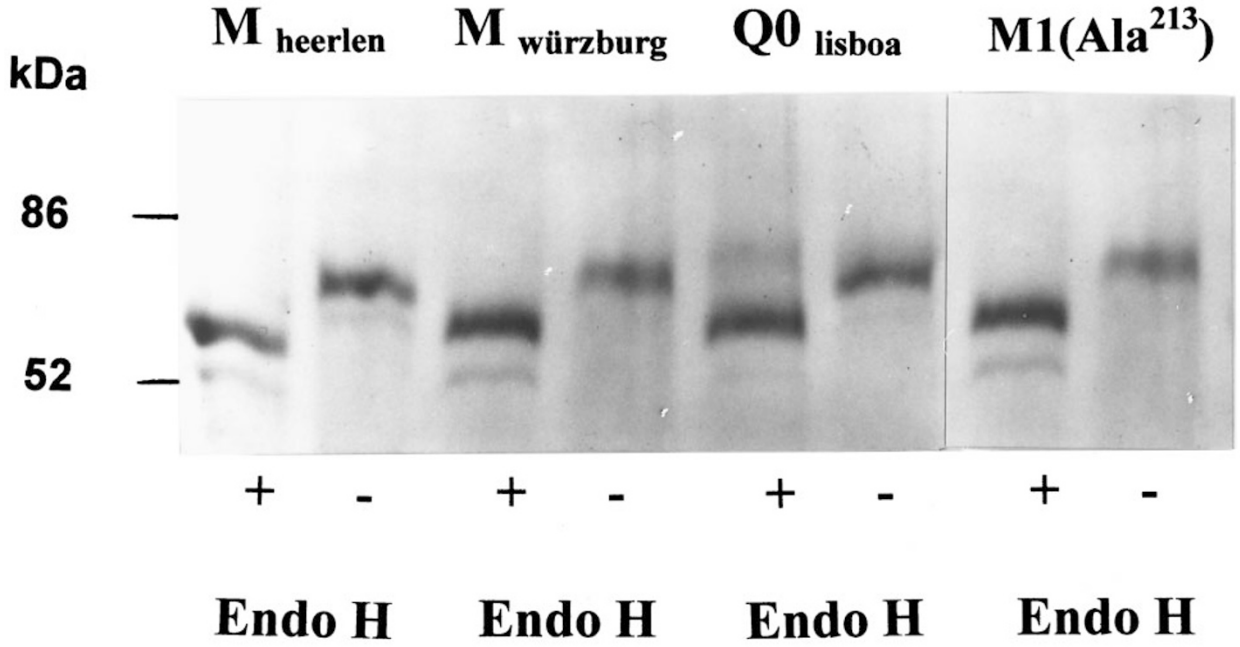

Figure 3 *Intracellular localisation of the transport block in the defective α1AT alleles: SDS-PAGE of the ^{35}S-labelled recombinant proteins Mheerlen, Mwürzburg, Q0lisbon, and wild-type M1 protein in the cell lysates is shown with (+) and without (–) Endo H pretreatment. The Endo H (–) lanes show the native intracellular proteins with N-linked glycosyl side chains attached. The reduction of size observed for M1 protein after Endo H treatment reflects the removal of high mannose and specific types of hybrid Endo H-sensitive glycosyl side chains added to the nascent protein during its transport through the rough ER. Proteins reaching the Golgi complex are further processed to contain complex hybrid side chains which are Endo H resistant. Although the Mheerlen and Mwürzburg alleles are retained completely within the cells they remain Endo H sensitive, suggesting that they cannot pass the ER which is obviously the intracellular site of the transport block for these defective proteins.*

positive intracytoplasmic globular inclusions were detected after diastase digestion.

Characterisation of Defects Associated with the PI Q0lisbon Allele

PI Q0lisbon protein was synthesised in similar quantity and apparent molecular size as normal PI M1 α1AT, but only a minor fraction of the mutant protein was secreted into the medium during the pulse phase (Figure 2, lane 2 in panel C). Although only a minor fraction of the mutant protein was secreted from the cells during the following 4 hour chase period, its intracellular concentration dropped markedly (Figure 2, lane 5 in panel C). This contrasts with the high stability of the intracellular Mwürzburg and Mheerlen proteins (Figure 2, lane 5 in panels A and B).

Discussion

We have characterised the molecular defects in three α1AT alleles, all of which are associated with anomalous intracellular protein transport. Pulse-chase experiments have shown that in two of them (PI Mwürzburg and Mheerlen) the quantity of synthesised α1AT is normal, but that the mutant proteins are completely retained within the cells as a consequence of a transport block between ER and Golgi complex. Adenovirus-mediated gene transfer to express both transportation-deficient mutants and wild-type α1AT in mouse liver showed that no deficient human a1AT was detectable in the mouse plasma, whereas high levels of circulating wild-type human α1AT were seen. The transportation defect in PI Mwürzburg and Mheerlen thus resembles that observed in the most common defective α1AT allele PI Z, although in the case of PI Z 10–15% of the synthesised protein is still secreted from the cell. The common Z mutation in exon 5 of the gene (Glu342Lys) causes disruption of the internal salt bridge between Glu342 and Lys290 which helps to stabilise the α1AT molecule. Neither this salt bridge nor the other between Lys397 and Glu264 is directly affected by the Mwürzburg and Mheerlen mutations, but both are localised in the C1 β-sheet region between Glu342 and Lys387 and might therefore change the

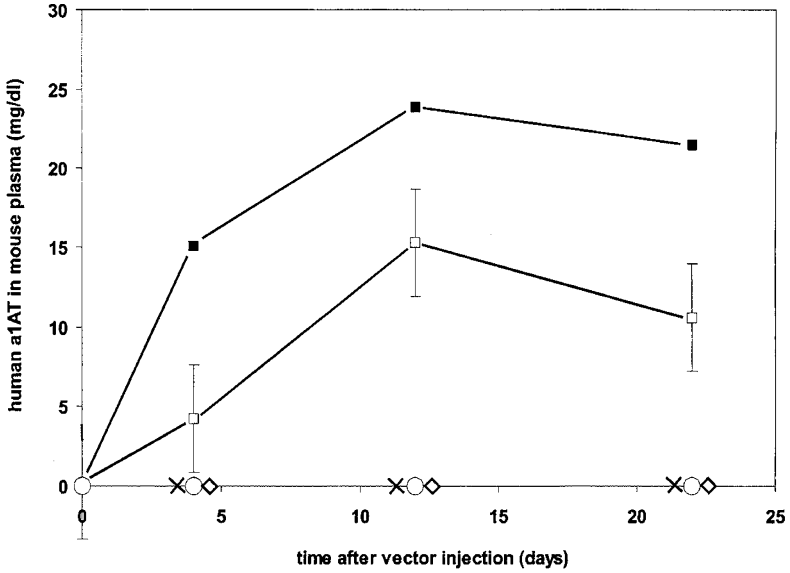

Figure 4 In vivo *expression of wild-type and mutant α1-antitrypsins: Shown are the levels of recombinant human α1AT circulating in the plasma of mice injected with adenoviral vectors expressing human wild-type α1AT, mutant PI Mwürzburg or Mheerlen α1AT, or luciferase, respectively. Vectors were injected into four groups of three mice each at a 'standard' dose of 2 × 10^10 particles per animal: wild-type (□), Mwürzburg (**X**), Mheerlen (◇), luciferase (○). Additional mice were given 'high' doses of 1 × 10^11 particles per animal of each vector type. Plasma α1AT levels for this dose are shown for wild-type vector only (■), since for the others no circulating human α1AT was detectable even when a 'high' dose was given. Human α1AT levels were assayed by using a strictly species-specific radial immunodiffusion assay detecting only human α1AT down to a concentration of 0.8 mM (4 mg/dl).*

conformation of the molecule in such a way as to indirectly impair formation of the salt bridges and thus proper folding and transportation of the protein. Since homozygosity for PI Z causes severe liver disease in a subset of patients,[7,38] this raises the question of hepatocellular damage in compound heterozygous carriers of the Mwürzburg or Mheerlen mutations in combination with the frequent Z allele, if these individuals are 'susceptible' to a1AT-induced liver disease.[21–23] Our patient with the Mheerlen mutation was heterozygous for the non-expressing null-allele Q0granite falls,[20,31] had extremely low α1AT serum level and severe COPD, but no evidence of liver disease. The heterozygous carrier of the Mwürzburg mutation also had no signs of liver injury. Although a long-term study of PI ZZ homozygotes has shown that 37% of these individuals ultimately suffer from liver cirrhosis and that 15% additionally develop primary hepatocellular carcinoma,[12] the question whether the PI Mheerlen and Mwürzburg alleles can trigger liver disease in PI Z compound heterozygotes remains to be answered. The key experiment towards this end will be expression of the mutant alleles in fibroblasts from PI ZZ patients with liver disease ('susceptible hosts' according to Perlmutter[21,22]) and from PI ZZ individuals without liver disease ('protected hosts').

In contrast to the complete transport block of PI Mheerlen and Mwürzburg, the molecular defect in the non-expressing allele PI Q0lisbon[25] is more complex. This mutant protein is synthesised in quantity similar to that of wild-type α1AT, but only about one third is secreted from the cell as compared with the wild-type. Interestingly, a mutation of the α1AT gene in the immediate vicinity of the Q0lisbon mutation has been described previously.[39] This defective allele PI Mmineral springs is caused by a substitution Gly67Glu. Similar to PI Q0lisbon (Thr68Ile), this mutation is also associated with reduced α1AT secretion from the cell. In addition, the secreted PI Mmineral springs protein is functionally deficient with impaired NEL inhibitory potential[39] which was not assessed in this study for PI Q0lisbon. The immediately neighbouring mutations Mmineral springs and Q0lisbon are located near the amino-terminal end of the α1AT molecule in the B α-helix. Therefore, the molecular mechanism responsible for intracellular protein accumulation in these two proteins should be distinct from the mechanism effective in the alleles PI Z or Mwürzburg or Mheerlen, but similar to each other. Our PI Q0lisbon index case was a child heterozygous for this defective and one normal PI M1 allele and had α1AT serum levels well above the protective threshold for pulmonary disease of 11 mM

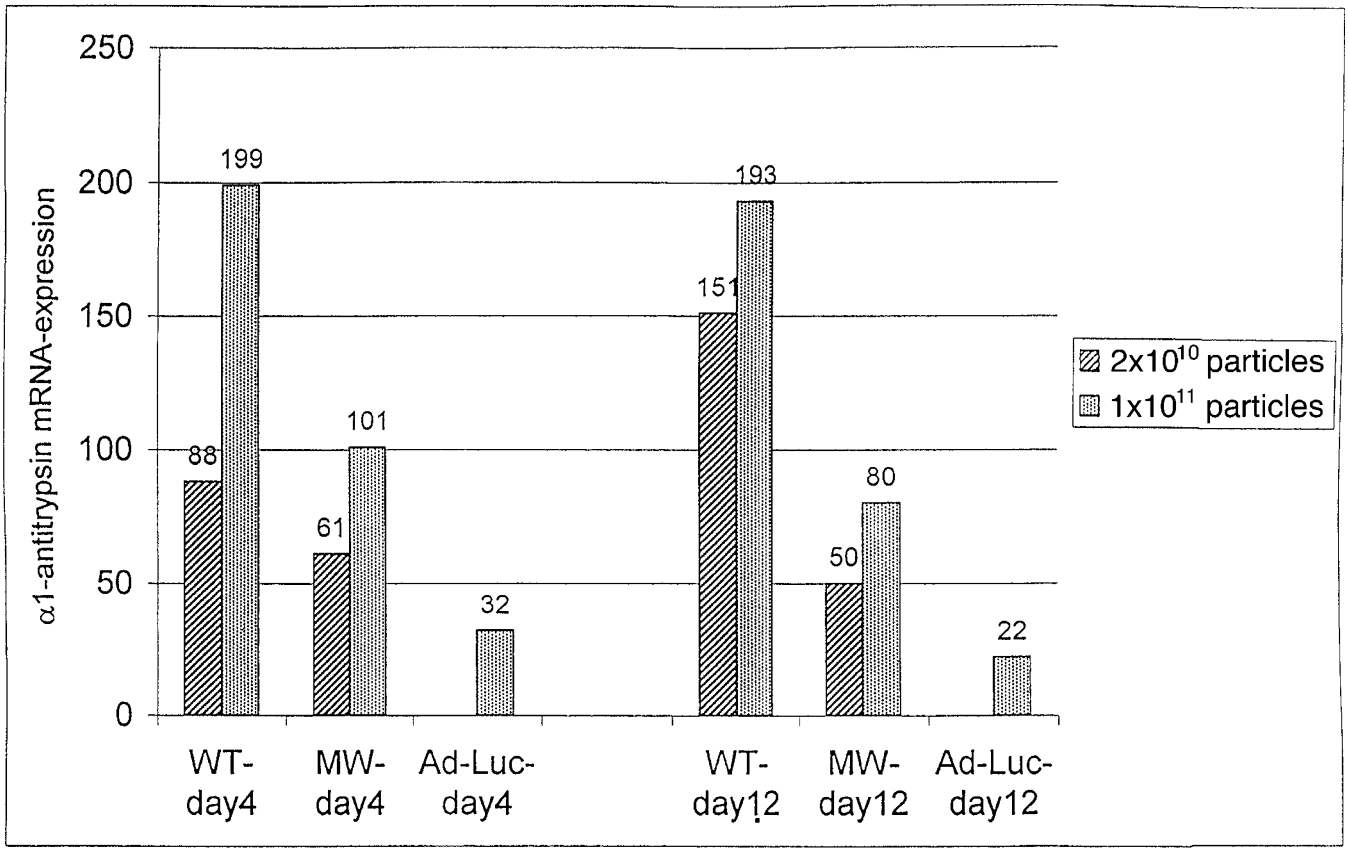

Figure 5 *Northern analysis of α1AT transgene expression in mouse liver. Shown are the steady-state α1AT-mRNA levels in the liver of mice injected with adenoviral vectors expressing wild-type α1AT (WT), mutant PI Mwürzburg (MW), or luciferase (Ad-Luc), respectively. Vector doses were 2 × 10^{10} and 1 × 10^{11} particles per animal, respectively. α1AT-mRNA expression was assessed by Northern analysis using a α1AT probe and a β-actin probe as internal standard. The calculated α1AT-mRNA levels (see Materials and Methods) are presented as mean values above the bars indicating the different vectors. Both wild-type and Mwürzburg α1AT vectors result in a significantly higher (up to 8.8-fold) hepatic α1AT-mRNA expression as compared with the luciferase control vector, indicating strong human α1AT transgene expression for both alleles. This contrasts with the high levels of secreted human wild-type α1AT versus non-detectable human mutant α1AT in the mouse plasma (Figure 4).*

(57 mg/dl), but lung disease could occur in homozygotes.

In summary, all defective α1ATs characterised here have the potential to cause lung disease in the homozygous state or in heterozygous carriers of another deficiency allele. The molecular mechanism of the transport block in two of these alleles (Mwürzburg and Mheerlen) may be similar to that in the PI Z protein. The molecular defect in the PI Q0lisbon allele shows similarity with the immediately neighbouring PI Mmineral springs mutation.

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