Immunisation of Chickens with the Aminoterminal Propeptide of Bovine Procollagen Type III
(Specificity of egg yolk antibodies and comparison with immunoassays using rabbit and mouse antibodies)

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Summary
Two chickens were immunised with the aminoterminal propeptide of bovine procollagen type III (PIIINP) purified from bovine fetal skin. Both animals developed antibodies binding to either unmodified or iodinated bovine PIIINP, but only one chicken developed antibodies which recognise PIIINP variants in serum. These antibodies were used to establish RIAs to analyse the specificity of these particular antibodies and to compare their specificity with that of published assays, as well as laboratory assay variants utilising different rabbit and mouse antibodies.

In comparison to most anti-PIIINP polyclonal antisera from rabbits the chicken antibodies do not bind to the Col-1 domain of PIIINP, a characteristic which they share with a monoclonal antibody (MAB 238) produced from a mouse immunised with the same antigen. Like the monoclonal antibody, the chicken antibodies exhibit reactivity against intact serum PIIINP and its high molecular weight variants, most probably pN procollagen type III and procollagen type III. While the monoclonal antibody can only be applied to analyse PIIINP in human sera, the avian antibodies show reactivity to the antigen in both, human and rat sera. The chicken anti PIIINP antibodies described in this study may become a powerful tool to quantitate PIIINP in serum of patients with liver fibrosis and in serum from experimental rat models of fibrogenesis. The fact that both can be analysed with the same assay, gives – for the first time – the opportunity for a direct comparison of the results from human and rat studies designed to evaluate the action of antifibrotic agents.

Keywords: radioimmunoassays, N-terminal propeptide of procollagen type III (PIIINP), avian antibody (IgY), liver fibrosis

Abbreviations: aa: aminoacid; IRMA: immuno radiometric assay; CFA: complete Freund's adjuvant; MAB: monoclonal antibody; PBS: phosphate buffered saline; pN procollagen type III: Procollagen type III lacking the C-terminal propeptide; RIA: radioimmunoassay.
1 Introduction

Collagen type III, like all fibrillar collagens, is synthesised in the form of a procollagen molecule containing C-and N-terminal extension peptides. These extension peptides are removed from the triplehelical molecule before its integration into a growing collagen type III fibril.

In fibrotic diseases of the liver, lung or skin, elevated serum levels of type III collagen propeptide (PIIINP) is believed to reflect the actual synthesis rate of type III procollagen and in particular the fibrogenic activity (Ruwart et al., 1989; Savolainen et al., 1988). At present, PIIINP seems to be a useful marker for monitoring the progression of fibrosis as well as the efficacy of antifibrotic drugs in both, clinical application and in diverse animal models of fibrosis (Wu and Danielsson, 1995; Schuppan et al., 1995; Hayasaka et al., 1991; Risteli and Risteli, 1990). However, the interpretation of the data from different publications is difficult because 1: PIIINP is present in serum in several antigenic forms with differing molecular weights, 2: the assays available make use of different antibodies with differing binding specificities to the various variants of PIIINP in serum, 3: there is no assay available which can be applied to measure intact PIIINP in both, human and rat sera (as the predominant species in animal models of fibrogenesis).

The present study describes two different new immunoasays for PIIINP which overcome these difficulties.

2 Methods

2.1 Materials

Buffers and reagents were obtained from Merck (Darmstadt, Germany) Sigma (Munich, Germany) or Riedel-de Haén (Seelze, Germany). They usually were of analytical grade.

2.2 Chromatographic separation of serum PIIINP antigen variants

To analyse the distribution of PIIINP in different sera, 1 ml of serum was passed over a Pharmacia SD 200 HR column (10/30) at a flow rate of 0.2 ml/min making use of a FPLC (Pharmacia). The column was equilibrated in PBS, 0.02% sodium asid, 0.04% Tween 20. The flow through was fractionated (0.5 ml fractions) and PIIINP was analysed in the fractions by different RIA variants.

2.3 Assays

PIIINP in human sera was measured using a competitive RIA as described by Rhode et al. (1979) (antisera specific: K-2), or by using an assay which is analogous to the coated tube assay RIA (Agost PIIP-IRMA established by Behringwerke Marburg and now distributed by CIS bio international (France). The coated tube assay applied in this study was performed according to the kit instructions with the identical standard, tracer (MAB 296) and buffers but the monoclonal coat antibody (MAB 226) of the commercially available kit was replaced by MAB 238.

PIIINP in rat sera was measured using a competitive RIA as described by Brocks et al. (1993), with a polyclonal antiserum against 14 aminoacids of the C-terminus of bovine PIIINP (antisera specific: K-PIC-14).

Radioimmunoassays with different polyclonal antisera at dilutions capable of 50% tracer binding (table 1), were performed as described by Brocks et al. (1993). Bound and unbound tracers were separated using magnetic antiserum, either to rabbit or chicken immunoglobulins (200 µl per probe; Paesel & Lorel, Hanau, FRG).

2.4 Epitope scanning

The epitopes recognised by the different antisera/antibodies were determined using a commercially available kit (Pin Technology™ Epitope Scanning Kit, Cambridge Research Biochemicals, Northwich, Cheshire, UK). 128 pins carrying peptides of 8 aa were synthesised. The first pin carried a peptide representing aa 19-26, the last pin represented aa 146-153 of bovine N-terminal propeptide of procollagen type III (Brandt et al., 1984), counted from the translation start site (Ala-Kokko et al., 1989). The pins were incubated with the respective antisera/antibodies. Bound antibodies were detected with species specific IgG conjugated to alkaline phosphatase (Sigma, Munich, Germany), employing an ELISA technique.

2.5 Preparation of PIIINP

PIIINP from fetal calf skin was prepared as described in Bruckner et al. (1978). The final purification step was carried out on a column of agarose A 1.5 M (2x120 cm, BioRad) equilibrated in 1 M CaCl₂, 0.05 M Tris-HCl (pH 7.5), or by reversed phase chromatography utilising a Bakerbond WP-C8 column (4.6x250 mm) equilibrated with 0.1% TFA. Bound antigen was eluted by applying a linear gradient of acetonitril (0-72%) in 0.05% TFA (data not shown).

3 Preparation of chicken antibodies

The chickens obtained from a commercial breeding farm (Geflügelvermehrungsbetriebe Spreehagen GmbH) were kept under artificial conditions (12:12) in cages appropriate for an approximate normal social behaviour. This cage type is recommended for chicken housing from the Swiss Veterinary Administration (Schweizer. Bundesamt für Veterinarwesen). The chickens were fed with a standard pellet diet for laying hens. Additionally, wheat was given on the cage floor to keep the chickens busy. The chickens were kept in groups of two animals coloured white and brown for egg identification.

Immunisation of chickens with bovine PIIINP was performed as follows: A volume of maximal 1 mL (0.5 mL antigen solution/0.5 mL CFA) containing approximately 150 µg of antigen was injected i.m. in the left and right Musculus pectoralis just before the chickens began to lay eggs. After four to six weeks a booster immunisation was performed. After about 10 days following the booster injection eggs were collected for antibody preparation. The egg yolks were diluted in destilled water (pH 7.2) 1:10, homogenised and stored for about 24 h at 20°C. After thawing the sediment was separated by centrifugation, the supernatant was used for further investigations.

4 Results

4.1 Specificity of polyclonal rabbit anti-PIIINP antisera

The immunisation of rabbits with bovine PIIINP usually leads to antisera with...
differing specificities to PIIINP variants in human serum. Most of the sera obtained from rabbits show a strong binding activity to the Col-1 domain of the antigen either integrated in the trimeric propeptide or as monomer (for illustration see insertion in fig. 2), but some rabbits also develop a minor population of antibodies with affinity to the telopeptide of PIIINP. This is schematically illustrated in figure 1, where a mixture of 7 polyclonal rabbit anti PIIINP antiserum (K-mix) is analysed for binding epitopes by making use of a pin technology. The mixture of the sera was preadjusted in order to obtain equal affinities after gelchromatography to PIIINP antigen variants in sera from patients suffering from liver fibrosis.

The size distribution of PIIINP in human serum separated by gelchromatography and analysed with a RIA using the rabbit anti PIIINP antiserum K-2 is illustrated in fig. 2. The antiserum specifically recognises about 10 aa at the C-terminal border of the Col-1 domain (fig. 1, Brandt et al., 1984). As can be expected, the main antigenic determinant in human serum, eluting at fraction 25 contains Col-1 (peak D). This is the predominant PIIINP-related antigen in serum that is believed mainly to originate from the degradation of pN procollagen type III which is incorporated in collagen III fibrils (Rohde et al., 1983b; Jensen et al., 1993). The high titered antiserum K-2 measures PIIINP antigenicity in control human serum with a content of PIIINP of 40 ng/mL and about 2-fold elevated concentrations in sera from patients suffering from liver fibrosis. This is summarised in the table which shows the characteristics of the different antibody preparations used in this study.

### 4.2 Specificity of monoclonal mouse anti-PIIINP antibodies

For human sera an assay is established which allows the specific determination of PIIINP and high molecular weight variants of the antigen. While the polyclonal anti PIIINP antibodies from rabbit and chicken bind to defined sequences of PIIINP in different domains of the antigen, the mouse monoclonal antibodies MAB 296 and MAB 238 do not clearly react with the oktapeptides synthesised on the pins (fig. 1). This indicates an exclusive conformation dependence of binding. An additional proof of the binding preference to natively structured intact PIIINP is the loss of PIIINP binding after denaturation and reduction of the disulfide bonds in the antigen (data not shown).

The sandwich assay IRMA-238 utilising MAB 238 to coat the assay tubes and MAB 296 as tracer detects approximately 3 ng/mL PIIINP in control human serum and about 3-5 fold elevated

### Table: Comparison of different antisera / antibodies with specificity to PIIINP

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dilution</th>
<th>%-binding of $^{125}$I-PIIINP $^2$</th>
<th>PIIINP in rat serum $^3$ ng/ml</th>
<th>PIIINP in human serum $^3$ ng/ml</th>
<th>% increase of PIIINP in liver fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-2</td>
<td>1:40000</td>
<td>50</td>
<td>-</td>
<td>40</td>
<td>150-250</td>
</tr>
<tr>
<td>K-P/C-14</td>
<td>1:850</td>
<td>50-60</td>
<td>10</td>
<td>-</td>
<td>-300</td>
</tr>
<tr>
<td>MAB 238 $^1$</td>
<td>$&gt;1 \times 10^4$</td>
<td>40</td>
<td>-</td>
<td>-3</td>
<td>300-500</td>
</tr>
<tr>
<td>MAB 296 $^1$</td>
<td>$&gt;1 \times 10^4$</td>
<td>40</td>
<td>-</td>
<td>4,5</td>
<td>300-500</td>
</tr>
<tr>
<td>Chick 1</td>
<td>1:500</td>
<td>50-60</td>
<td>5</td>
<td>4,5</td>
<td>200-400</td>
</tr>
<tr>
<td>Chick 2</td>
<td>1:1000</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$ from ascites; $^2$ because it was intended to apply the antisera in radioimmunoassays, it was tried to obtain a 50% binding of iodinated PIIINP (1 nM). $^3$ The sera were tested with respect to binding to PIIINP in rat and human serum (N > 10) in a RIA with the exception of MAB 238 which was applied in a sandwich assay with MAB 238 coated wells and MAB 296 as tracer (IRMA-238).

![Figure 1: Determination of antigenic epitopes in PIIINP with different antibody preparations.](image)

128 pins carrying peptides of 8 aminoacids were synthesised, covering the entire length of PIIINP. The first pin carried the sequence QEQADVGG, the last peptide synthesised on a pin (no. 128) showed the sequence GGCNYSPQ. The peptides used for epitope scanning are represented on the x-axis by their numbers. Antibody binding was detected by an ELISA technique using species specific antibodies coupled to alkaline phosphatase. The affinity to the peptides is illustrated by the thickness of the bars. In the case of the monoclonal antibodies 296 and 238 the positive reaction, indicated by the thin lines, was only petty elevated over the blank control.

chick 1: denotes a polyclonal chicken IgY. MAB 296: denotes a monoclonal mouse antibody used as tracer in RIAnosert PIIINP - IRMA and IRMA-238. MAB 238: denotes a monoclonal mouse antibody used to coat the vials in the laboratory assay variant IRMA-238. K-mix: denotes a mixture of polyclonal rabbit antiserum. K-P/C-14: denotes a polyclonal rabbit antiserum with specificity to the 14 C-terminal aa of PIIINP (Brocke et al., 1993). K-2: denotes a polyclonal rabbit antiserum with specificity to a specific aa sequence in Col-1.
vated levels in sera from patients with liver fibrosis. As shown in table 1, there is no cross reactivity to rat PIINP. The low amount of PIINP in serum samples of healthy human individuals reflects the lack of Col-1 recognition with the monoclonal antibodies, which is also clearly visible after gel filtration of a serum from a patient with alcoholic liver fibrosis (fig. 2.2). The peak eluting immediately after the void volume of the column (peak A, > fraction 9) most likely is procollagen type III and/or pN procollagen type III. The peak eluting at fraction 19 (peak C) is PIINP, because at this position the standard bovine PIINP elutes in a symmetrical peak (not shown). The antigenic variant eluting from the column at fraction 16 has twice the molecular weight of PIINP (peak B) and is believed to derive from a transglutaminase related dimerisation of PIINP or crosslinking to fibrinogen or low density lipoprotein (Bowness et al., 1990, 1989). The fact that assays using the mouse monoclonal anti PIINP antibodies either in a RIA with MAB 296 or in a sandwich configuration with MAB 296 and MAB 238 measure 3–5 fold elevated serum levels of PIINP in patients with liver fibrosis indicates a higher contribution of changes in intact PIINP variants in fibrotic conditions as compared to Col-1.

4.3 Specificity of polyclonal rabbit antibodies against a specific peptide sequence in PIINP
A RIA using the polyclonal antiserum K-P/C-14 directed against a synthetic peptide representing the 14 C-terminal aminoacids of rat and bovine PIINP (Brocks et al., 1993; fig. 1) was developed to quantitate native PIINP. Unexpectedly, this assay does not detect human PIINP but it is very well suited to analyse intact PIINP in rat sera. This is explained by the authors as due to the Gly / Pro exchange at position 148 (Brocks et al., 1993; Wood et al., 1987; Ala-Koko et al., 1989). The assay does not recognise Col-1 and is believed to measure exclusively type III procollagen synthesis. The RIA making use of this particular antiserum is capable to determine about a 3-fold elevation of serum PIINP in rats with experimental liver fibrosis (table). A comparison of the PIINP size distribution in sera from CCl4 treated rats and from a patient with alcoholic liver fibrosis (analysed with IRMA-238) reveals a high degree of similarity, but no identity of the PIINP patterns in both species (fig. 2.2 and 2.4). The question whether this is due to different affinities of the different antibodies to serum PIINP

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Figures 2-6: Analysis of the size distribution of serum PIINP with different immunoassays after gel filtration. Different immunoassays for PIINP antigens in human and rat sera were applied as indicated at the top of the chromatograms. The identification of different PIINP variants is given in the chromatograms. The fraction numbers are indicated on the x-axis. For a better comparability the relative amount of PIINP variants is given in arbitrary units. A: denotes pN procollagen type III or procollagen type III. B: denotes crosslinked PIINP (see text). C: denotes intact PIINP. D: denotes Col-1 fragment. For better understanding, a scheme depicting the domain structures of procollagen type III and PIINP is inserted.
variants in rats and man to varying amounts of the high molecular weight forms of the antigen in rat and human sera cannot be answered unambiguously.

4.4 Specificity of polyclonal chicken antibodies against PIIINP

To produce antisera with altered affinities we immunised chickens with bovine PIIINP. Hen's egg is known as an efficient source of IgY antibodies (Schade et al., 1991; Lösch et al., 1986) and represents an alternative for the production of immunoreagents for the determination of different components in biological fluids (Lee et al., 1991; Vieira et al., 1984). In addition the production of avian IgY can be a successful approach to obtain antibodies with new specificities, especially in the case of strictly conserved mammalian antigens (Gassmann et al., 1990). The two chickens immunised developed antibodies 5 weeks after immunisation which were capable of 50% tracer binding at yolk dilutions up to 1:1000 (table 1), but only the antibodies from one chicken (chick 1) were able to bind to serum PIIINP or to become displaced from iodinated bovine PIIINP in a RIA after incubation with serum, respectively. To our big surprise, the chicken antibodies showed affinity to PIIINP in both, human and rat serum as analysed with a RIA using the chicken IgY and bovine PIIINP as tracer and standard.

The epitope analysis clearly shows that the chicken antibodies, like the rabbit anti peptide antisera K-P/C-14, predominantly recognise the 10 last aa in the telopeptide of PIIINP with additional low affinity sites in the triplehelical domain and in Col-I (fig. 1). However, PIIINP binding is not strictly sequence specific because the antibodies cannot be displaced from iodinated bovine PIIINP by peptides covering the 14 C-terminal aa of bovine PIIINP at concentrations below 1 μg/mL (data not shown). An additional indication for the conformation dependence of PIIINP recognition is the fact that the avian antiserum is not able to bind to bovine PIIINP simultaneously with MAB 238 which has no strongly marked affinity to the aminocacid sequence on the telopeptide (fig. 1). Probably both recognise a conformation in which the C-terminal aa are included or which is determined by the 14 aa representing the C-terminal end of the PIIINP sequence.

The concentration of PIIINP monitored with the „chicken assay“ in rat and human control sera is about 5 ng/mL. In fibrotic conditions the amount of PIIINP rises about 3 fold in both species (table). In figures 2.3. and 2.5., the size distributions of serum PIIINP in „fibrotic“ human and rat sera are illustrated. In both species the chicken antibodies do not identify the Col-1 domain as could be expected from the epitope analysis. However, they strongly bind to high molecular weight PIIINP forms (pN procollagen type III and procollagen type III). The intact PIIINP and its presumed transglutaminase related modification are identified in different quantities in human and rat serum. However, this observation has to be validated by performing additional measurements with a higher number of sera either from patients with different causes of liver fibrosis or fibrotic animals treated with different toxins. The fact that the same PIIINP variants with identical molecular weights are identified in both sera points to an identical fate of PIIINP in rats and humans or to the same mechanism of clearance from the circulation, respectively.

5 Discussion

In studies elaborated to determine fibrosis activity in liver or to monitor the inhibition of fibrosis after treatment with specific drugs, assays are needed that mirror the synthesis of collagen which is correlated with the amount of intact PIIINP in serum (Jensen et al., 1993; Hayasaka et al., 1991; Ruwart et al., 1989; Savolainen et al., 1988). The cleavage of PIIINP from pN procollagen type III is an intermediary step for type III collagen fibril formation and, consequently, serum PIIINP is suggested as surrogate parameter for fibrogenesis (Risteli and Risteli, 1995). The intact N-terminal propeptide of collagen type III is a trimer of identical subunits, containing globular domains (Col-1), a short triple helical domain and a peptide extension (about 14-20 aa) at the C-terminal side (Niemelä et al., 1985; Nowack et al., 1976) with unknown structure (ref. insert in fig. 2).

The immunisation of rabbits results in highly potent antibody preparations like K-mix or K-2 which are characterised by a strong reactivity to the Col-1 domain in human serum. A comparable pattern of antigen distribution in human serum may be obtained with the well known human specific radioimmunoassays for PIIINP, the previously commercially available RIAgnost® PIIINP-Fab (Behringwerke, Germany) and the monoclonal mouse antibody based RIAgnost® PIIINP-IRMA (CIS bio international, France; formerly Behringwerke Radiodiagnostics). The liberated Col-1 fragment present in serum is believed to derive from the degradation of type III collagen fibrils, hence all the above mentioned immunoassays are considered to reflect fibrolysis due to their given affinities to the Col-1 fragment (Risteli and Risteli, 1995; Rhode et al., 1979 and 1983a, b; Niemelä, 1982).

Replacing the capture antibody of the commercially available RIAgnost® PIIINP-IRMA by mouse MAB 238 results in an assay that exclusively measures intact PIIINP and its high molecular weight forms as could be shown in this study. Indeed, the assay IRMA-238 is a diagnostic tool to monitor active collagen synthesis or liver fibrogenesis in human sera (unpublished observations), respectively. But unfortunately, the assay can not be applied for the determination of intact PIIINP in rat studies of fibrogenesis or inhibition of fibrosis. This is a disadvantage because a definite proof of PIIINP as a surrogate reflecting the actual synthesis rate of collagen (fibrogenesis) can not be approached experimentally by using the improved IRMA-238.

This drawback could partly be avoided by the establishment of a RIA utilising polyclonal rabbit antibodies directed against a synthetic peptide deduced from rat and bovine PIIINP (Brocks et al., 1993) (fig. 1). Like the IRMA-238, this assay does not recognise Col-I and is believed to measure exclusively type III procollagen synthesis. Unexpectedly, the RIA does not react with human PIIINP and the pattern of PIIINP variants in rat serum only is comparable but not identical to...
the pattern obtained with IRMA-238 in human serum. In consequence, a direct comparison and extrapolation of the results obtained from rat studies related to liver fibrosis to the situation in humans still remained problematic and could only be performed with caution.

The chicken anti-PIIINP antibodies described in this study allow for the first time the measurement of identical defined PIIINP variants in rat and human sera with the same immunoassay. The interpretation of results is not affected by influences from a cross reactivity to the liberated Col-I fragment, thus the ‘chicken assay’ fulfills the criteria for a diagnostic tool for the assessment of collagen type III synthesis or active fibrogenesis. This holds in particular for human serum, where the predominant PIIINP variant that can be detected is PN procollagen type III or/and procollagen type III.

Future studies are planned which are addressed to the questions of: 1: synthesis of collagen type III in rats and humans with ongoing fibrogenesis, 2: monitoring antifibrotic effects of prolyl 4-hydroxylase inhibitors and 3: comparison of species with respect to PIIINP diagnostics with a RIA utilising the chicken antibodies.

References


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