Celiac disease : towards new therapeutic modalities

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Chapter 6

Discussion

DISCUSSION

Currently the only treatment for celiac disease is a lifelong gluten-free diet. While very effective it is cumbersome as wheat based products are so commonly used in our daily diet. Many food products are therefore off limits for celiac disease patients. Moreover, gluten is often present as a hidden ingredient which can lead to inadvertent exposure to gluten. While many patients are content with their gluten-free diet, others would welcome alternative treatments and/or food products that would allow more flexibility. The research presented in the present thesis focuses on the development of such alternatives.

Celiac disease is an HLA-associated disease: it develops almost without exception in HLA-DQ2 and/or HLA-DQ8 individuals (1-3). There are many other HLA-associated diseases, like type I diabetes and rheumatoid arthritis, but only in celiac disease the disease-causing agent is known: wheat-derived gluten and similar proteins in other cereals. Around 1950 Willem Karel Dicke first observed the connection between cereal intake and the presence of symptoms in children with celiac disease (4). He found that during the Second World War, when wheat products were scarce, his patients got better but relapsed when wheat was reintroduced after the war. Later research demonstrated that the symptoms were caused by the gluten and gluten-like storage proteins in wheat and related cereals (5). Based on these observations the gluten-free diet was introduced: when a patient does not consume wheat, barley and rye products she/he does not experience any symptoms. The definition of a treatment is that it treats a problem and may lead to its cure but more often the treatment ameliorates a problem only for as long as it is applied. This is also the case in celiac disease, the gluten-free diet offers release of symptoms only as long as it is followed and therefore must be maintained lifelong. It's main disadvantage is that it can restrict participation in social events, traveling and eating out which causes more problems to the newly diagnosed and to young patients.

In the present thesis three alternative approaches to the gluten-free diet are presented and discussed. These approaches are: the development of cereals or gluten with no or low immunogenic content, the use of oats as an alternative cereal, and the use of enzymes for gluten detoxification.

Better detection methods

In order to determine gluten toxicity, appropriate reagents need to be available to detect such toxicity. Earlier studies had defined the epitopes responsible for the immune response. These were found to be peptides derived from both gliadins and glutenins (6-9). All such peptides have been identified with the use of patient derived T cells specific for such peptides when bound to the disease predisposing HLA-DQ2 or HLA-DQ8 molecules. Therefore, the golden standard for testing the immunogenicity of food products would be the use of such gluten specific T cells isolated from the small intestine of celiac patients. Unfortunately, they can not be used for routine screening purposes as they are highly sensitive for various compounds commonly found in food,

can only be employed in a sterile environment and testing is time consuming and expensive. For this reason antibody-based systems are commonly employed. The validated gluten detection method currently employed is based on the R5 monoclonal antibody that was raised against a barley epitope and also reacts with sequences from gliadins and rye (10,11). Unfortunately, the epitope recognized by the R5 antibody does not represent the known toxic sequences of gluten and therefore does not measure gluten toxicity. Ideally, antibody-based gluten detection systems would employ antibodies that are raised against known T cell stimulatory sequences in gluten and have a reactivity pattern that closely resembles that of gluten specific T cells.

For this purpose five monoclonal antibodies were raised in our laboratory that detect known gluten epitopes namely: Glia-alpha-20, Glia-alpha-9, Glia-gamma-1, the LMW-glutenin 156 and the HMW glutenin epitope (12-14). These antibodies were used to set up a competition based assay that can detect the presence of these epitopes both in peptides mixtures and proteins. All 5 antibodies were shown to be suitable for gluten detection in Western blot analysis as well. The fine specificity of these antibodies for gluten and gluten-like peptides and proteins was determined and compared with that of the gluten specific T cells (Chapter 2). It was shown that, just like the gluten specific T cells the antibodies react with homologous peptides found in epitopes from barley, rye and triticale and that the specificity partially overlaps with that of the corresponding T cells (15). Such antibody reagents are therefore very suitable for the detection of gluten, in particular sequences that are linked to gluten toxicity for celiac disease patients. Therefore these antibodies were used in the subsequent studies aimed at the development of alternatives for the gluten-free diet.

Moreover, in collaboration with the diagnostics company Europroxima, one of the antibodies has been used to generate a gluten detection kit that has been tested in an international RING-trial in 2009/2010 in which 10 laboratories participated. As the results of this trial were positive and the new kit has several advantages over the commercially available R5 test kit, it was launched on the market in June 2010 and may ultimately replace the R5 based method.

Towards low toxic wheat and safe gluten proteins

As wheat is so important for food production in industrialized countries, one would like to have a wheat variety available that would be suitable for consumption by celiac disease patients. With the identification of the immune stimulatory sequences in the gliadins and glutenins it became possible to investigate if differences exist between wheat varieties or gluten proteins with regard to toxicity for celiac disease patients. Indeed, it could be shown that natural variation exists in the immunogenic content of wheat varieties, especially between varieties belonging to different genotypes (14,16). This sparked the hope that a non-toxic hexaploid bread wheat variety could be identified. The benefits of a non-toxic hexaploid wheat are obvious as they have good crop yield and good baking quality and could thus be easily used in the food industry. Ideally, this would form the basis for the production of food products that would have the same nutritional value and the smell, texture and taste typical for normal wheat based products. Further research, however, indicated that it is highly unlikely that natural wheat varieties exist that are safe for consumption by patients. The analysis of over

3000 gliadin genes indicated that none encoded a protein that would be entirely devoid of toxicity. Moreover, all ancient wheat varieties that we tested proved to contain immunogenic epitopes, although to a lesser extent than the hexaploids bread wheat varieties.

The results, however, offered an alternative approach to eliminate gluten toxicity (Chapter 3). Extensive testing of all existing variants of known immunogenic peptides in gluten proteins indicated that some of these variants lacked immunogenic properties. For example, a variant peptide present in the A genome contains one single amino acid substitution that rendered the epitope non-immunogenic. This single amino acid substitution concerned the replacement of the proline at relative position 8 in the peptide by a serine. Interestingly the majority of HLA-DQ2-restricted gluten epitopes contain a proline at this position (17) moreover, we observed that the introduction of this proline to serine substitution eliminated the immunogenic properties of all these peptides, including the highly immunogenic 33-mer found in the alpha-gliadin proteins. We used this knowledge to device a general strategy to eliminate gluten toxicity in alpha-gliadin proteins and provided proof of principle at the peptide level. Similar approaches are likely to be applicable to other gluten proteins as well. These results can now be applied and may lead to large scale production of safe gluten proteins for incorporation in “gluten-free” foods meant for celiac disease patients.

Thus, in our search for a non-toxic cereal we failed but we did find a way to detoxify gluten.

Alternative cereals: oat

While it is well established that wheat, barley and rye can not be tolerated by celiac disease patients, oat is considered a reasonable safe alternative. In the last 10 years, however, the introduction of oat in the gluten-free diet was surrounded by controversy since not all patients tolerate oat (18). “Oat toxicity” was partially explained by the discovery that the large majority of oat samples were contaminated with wheat, barley or rye during cultivation, transport and/or processing (19). To overcome this problem contamination-free oat chains have been set up in Scandanavia and more recently also in The Netherlands. Another problem surrounding oat is that it was found that gluten reactive T cells can cross react with peptides derived from the gluten-like avenin proteins in oat and the isolation of avenin specific T cell clones from a celiac disease patient that was intolerant to oat (7,20). Thus, oat does contain some peptides that can elicit immune responses but the level of exposure is apparently so low that most patients can consume oat without problems. To determine if all oat varieties are equally immunogenic we tested a representative collection of oat samples used in the Netherlands (Chapter 4). We observed that all oat samples tested contain immunogenic epitopes but that their presence is variable. Thus, just like in wheat, also in oat there is a natural variation in the immunogenic content. We also identified a monoclonal antibody which specifically detects avenin epitopes, the use of which will make the testing for intrinsic toxicity easier. The results indicate that though testing of the immunogenicity of oat, low or non-toxic varieties may be identified that will allow oat consumption by all celiac patients without any constraint.

The use of enzyme therapy for gluten detoxification

Oral supplementation with enzymes in order to diminish the exposure of patients to hidden gluten was proposed already in the late 1950 when was thought that celiac disease was due to a lack of enzymes (21,22). The first enzyme of choice was papain, but the results of the experiments were poor (22). The following attempt was performed with an extract from animal intestine (23). This clinical trial did show a certain protection in comparison to the placebo, but not all patients benefited to the same extent. Since the high proline content of gluten is one of the reasons for its poor degradation in the gastro-intestinal tract the use of post-proline cutting enzymes was proposed (24-26). Promising enzymes that were tested are the prolyl oligopeptidases from Flavobacterium meningosepticum, Sphingomonas capsulate and Myxococcus xantus, which were shown to cut proline rich gluten proteins (25-28). These enzymes, however, have a drawback since they can not function in the acid milieu in the stomach and can also be degraded by pepsin. They are thus not able to degrade gluten before it reaches the upper part of the small bowel where the characteristic celiac disease lesions are found.

An alternative enzyme is the prolyl endoprotease of *Aspergillus niger*, AN-PEP. It was already shown that this enzyme has several advantages over the previously described enzymes since it is more efficient in degrading gluten peptides, is active at a low pH and is resistant to pepsin degradation (29). During my research we took the testing of this enzyme to the next level and we showed that AN-PEP is capable to degrade gluten present in a simple bread meal and also in a more complex bread containing meal in a system that mimics the human gastro-intestinal tract (Chapter 5). The samples recovered from the “digested” meal were found to no longer contain immunogenic sequences when tested with specific monoclonal antibodies and more significantly with gluten specific T cells. These promising results form the pre clinical phase of a clinical trial which has already shown that the enzyme can be safely consumed by patients (Table 1). A larger clinical trial will have to demonstrate that the enzyme is useful for gluten degradation in humans and can prevent the reappearance of symptoms when patients consume gluten.

Table 1. Description of AN-PEP clinical trial

Phase	Description	Results
pre clinical	*In vitro* experiments	Effective degradation of known epitopes in food products
I	safety, pharmacokinetics, administration way	no side effects by oral administration
II	determination of optimal dose	not performed
III	comparison to known therapies, testing combination therapies and different doses	not performed
IV	long term efficacy and effects	not performed

The future: from bench to the patient

The best reward for any research is to see it applied. The focus of my research has been on the development of alternatives to the current gluten-free diet. Parts of the results presented in this thesis are closer to application than others. The detection of immunogenic gluten and gluten-like epitopes with the use of monoclonal antibodies is closest to being exploited and the anti-Glia-alpha-20 antibody is already incorporated in a detection kit which has been successfully tested in a ring trial. Since this kit detects a non-repetitive immunogenic gluten epitope and uses a synthetic peptide as a standard, it offers a better gluten quantification method compared to the existing method based on the R5 antibody. The kit was launched on the market in June 2010. While this kit is primarily meant for industrial use, it may be possible to design a variant that would allow patients to test food products themselves.

Also, the results on oat toxicity can be readily applied to ensure safety of oat consumption by patients. Unfortunately, the situation is much more complex when it comes to wheat. Large scale screening of gliadin genes indicated that non-toxic gliadin proteins do not exist. Accordingly, all tested hexaploid, tetraploid and diploid wheat varieties were found to contain toxic epitopes. It is thus highly unlikely that true non-toxic wheat can be obtained through conventional breeding programs. However, during these screening programs we discovered naturally occurring amino acid mutations in gliadin epitopes which eliminate the T cell stimulatory properties of these epitopes. This now allows the design and expression of modified gliadin genes for the production of non-toxic gluten. As the introduced amino acid modifications are naturally occurring it is expected that the modified proteins will have retained much of the desired technological properties and nutritional value of the original gluten proteins. Likewise, a similar detoxification method could be used to eliminate the toxicity of the gamma–gliadins, LMW- and HMW-glutenins, allowing the production of complex but non-toxic mixtures of gluten proteins. Although this approach is straightforward, the realization of “Safe Gluten” is still not around the corner. The genetic code of gluten genes will have to be changed and expressed in recombinant fashion, creating genetically modified crops producing recombinant gluten proteins. Apart from public acceptance and regulatory issues, it is unknown how efficient the production would be and it remains to be demonstrated that such “Safe Gluten” is indeed safe for consumption by patients. It will be several years before this point is reached.

Gluten with low dose epitope content can be obtained on a much shorter term either through selection of varieties with low epitope content of by specific amino acid mutations at key positions in known epitopes. By replacing gluten with this “lower epitope dose” gluten the risk of development of celiac disease may be lowered resulting is a reduced incidence of the disease.

The introduction of enzymes for gluten detoxification may be much closer at hand. After demonstrating that AN-PEP can degrade gluten in an artificial gastrointestinal system, the enzyme was tested in a small group of patients which showed that its consumption is not associated with adverse symptoms. Next it will have to be tested if AN-PEP can offer patients good protection against immunogenic wheat present in the diet. For this a large scale clinical trial needs to be executed. At present it is not clear if

the enzyme can offer protection against all wheat containing products since it needs to have access to the gluten in the food matrix and it is likely that this differs between food products. Therefore, it is unpredictable if AN-PEP will ever be able to replace the gluten-free diet. However, considering that AN-PEP has excellent gluten degrading capacity *in vitro*, it is likely that AN-PEP can be applied to safeguard celiac disease patients against unwanted gluten intake, for example during traveling or eating out.

Future prospects

While Dicke recognized already around 1950 that wheat and related cereals were causing celiac disease, it was not until the end of the last century that the molecular basis for this was unraveled. Now we know that gluten contains a multitude of immunogenic peptides that can be modified by tissue transglutaminase and bind to the disease predisposing HLA-DQ2 or -DQ8 molecules and trigger inflammatory T cell responses. Although much is still unknown – i.e. why do not all HLA-predisposed individuals develop disease and what triggers inflammatory responses in the epithelium? – we can try to exploit the current knowledge to the benefit of patients. This was the goal of the work described in the present thesis. In particular it was investigated if it would be possible to develop wheat or gluten proteins that would be safe for consumption by patients and if it is possible to enhance gluten degradation with a post-proline cutting enzyme isolated from *Aspergillus niger*. With the development of safe wheat patients would be able to consume a "normal" diet. Moreover, such wheat could be used to prevent the development of celiac disease in children born in high risk families. On the other hand, co-administration of a gluten degrading enzyme with a gluten-containing meal might allow patients to consume such meals. These two approaches are thus seemingly each others enemy: safe wheat would eliminate the need for enzymatic degradation while an effective enzyme therapy would eliminate the need for safe wheat. In reality the truth lies in the middle. There is no safe wheat and the development of safe gluten is still several years away from actual realization. It is also largely unknown how cost effective such safe gluten proteins can be produced and incorporated in foods for patients. On the other side it is unclear if enzymatic degradation of gluten in the gastrointestinal tract will be so effective that it will allow the introduction of a normal gluten containing diet. Thus, both options are still open and deserve to be explored further.

Next to better treatment options for patients, prevention of celiac disease development is an ultimate goal of research in this area. Given the importance of cereal-based products for nutrition worldwide exposure to gluten is unlikely to change significantly in the future. Reduction of the incidence of celiac disease can thus only be achieved by targeted intervention in high risk families. Delayed introduction of gluten and deliberate exposure to low amounts of gluten early in life are two approaches that are currently being tested. Ultimately, better markers for prediction of the risk of disease development will be required for the design and implementation of more specific intervention protocols. While genetic studies are rapidly identifying such markers, these studies also demonstrate that many gene variants are implicated, each with a low impact. The unraveling of this complex genetic picture will take many years, years in which a gluten-free diet will likely remain the safest option for celiac disease pa-

tients. Nevertheless, it is reasonable to assume that the work described in this thesis will in the near future contribute to the development of novel approaches in celiac disease management.

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