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CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

Inflammatory Bowel Disease is the common denominator for two clinically recognized disease entities: Crohn's disease (CD) and Ulcerative Colitis (UC), which are chronic inflammatory disorders of the gastrointestinal tract. The exact etiology of IBD is still largely unknown, but a general consensus is emerging that disease in humans is mediated by an uncontrolled immune response to the normal non pathogenic constituents of the gut.

Early epidemiological and family studies have made it clear that there is a strong genetic predisposition to develop IBD, in particular CD. The most compelling evidence for this came from twin studies which showed that concordance rates in identical twins with CD are as high as 58%, while concordance rates in dizygotics are similar to normal siblings. However the fact that the concordance rate in monozygotic twins is not 100% makes it clear that also other, environmental factors are involved. In addition, these studies revealed that the genetic component of the disease is not based on simple mendelian inheritance, but instead points to a complex involvement of several genes, distributed on different chromosomes.

The results from genome wide linkage and association studies that have become available in the past years have added enormously to our knowledge on the genetic complexity of the disease by identifying more and more potential candidate genes with a number that is now exceeding 50. However it should be kept in mind that not all these genes are involved in each patient. Disease is the combined result of several genes, each with its own larger or smaller defect, and modulation of these gene defects by environmental factors such as life style or composition of gut flora. This differential contribution of causative genes and external influences is reflected in the large clinical heterogeneity in age of onset, localization, or the appearance of concomitant diseases, typical for IBD. Identifying the underlying genetic defect and the mechanisms by which these genes exert their effect will ultimately lead to better treatment, and possibly even a cure of the disease.

Several of these aspects have been reviewed in more detail in **chapter 1**.

A valuable tool in unraveling this complex entity of interacting factors are animal models. Advantages of the use of experimental animals is that the disease is well defined because of the identical and often well-defined genetic background of the animals, in particular mice. In addition environmental factors, such as diet and use of drugs can be controlled, and once a gene is identified its function can be studied in more detail knockout or transgenic models.

Although an obvious drawback lies in the translation of results from animal data to the human situation, much of what we know now about IBD comes from the systematic analysis of animal models.

In several studies underlying this thesis we made use of the trinitrobenzene sulfonic acid (TNBS) inducible colitis model. Previously it was found in crossing studies that susceptibility to TNBS colitis as seen in the SJL/J strain, or the resistance associated with the C57Bl/6 strain, co-segregated with two loci [1]. One locus was positioned on chromosome 9, the other on chromosome 11. One of the genes located in the region of chromosome 11 codes for interleukin-12 (IL-12) p40, a subunit of two important pro-inflammatory cytokines, IL-12 and IL-23. The importance of this subunit can be inferred from the key observation that development of colitis in this model can be prevented or even reversed by using antibodies against the interleukin 12p40 subunit.[2].

In **chapter 2** we explored whether the IL-12p40 gene is indeed involved in susceptibility to SJL/J colitis and investigated the possible mechanism underlying this involvement.

Challenging SJL/J en C57Bl/6 mice with LPS, a well-known antigen from bacterial residents of the gut, showed a significantly higher IL-12 response in SJL/J mice than seen in C57Bl/6, which could be diminished by co-injection of IL-10, a strong anti-inflammatory cytokine. This showed that susceptible mice reacted with an excessive proinflammatory response when compared with the resistant strain, further supported by the observation that also the induction of TNBS colitis was characterized by elevated IL-12 responses in the susceptible strain. Further support for the actual involvement of IL-12p40 comes from an earlier study [1] where it was shown that the more elevated IL-12 responses upon challenge with LPS had a genetic component and that the locus associated to this higher response resides in the same chromosomal region where the gene for IL-12p40 is located.

As the mature bioactive cytokine IL-12 (IL-12p70) consists of two subunits (IL-12p40 and IL-12p35) which are covalently bound by two S-bridges, we expected this higher IL-12p70 production to be accompanied by a higher IL-12p40 production. However this appeared not to be the case since in serum the IL-12p40 levels were the same in both susceptible and resistant strains after challenge with LPS. Alternatively, the higher IL-12p70 synthesis could be attributed to a higher IL-12p35 synthesis. This is not very likely since genetic studies did not reveal a correlation with a region on chromosome 3 where the gene for the IL-12p35 gene

is located, and in addition, IL-23 which is formed by the covalent ligation of the IL-12p40 subunit to another subunit, p19, shows the same preferential formation of mature IL-23 with the SJL/J IL-12p40 chain. Together these data pointing to an inherent characteristic of the p40 subunit (this work chapter 2), e.g. resulting in a higher affinity of one subunit for an other as may occur as a consequence of amino-acid changes inducing spatial rearrangements energetically favoring subunit binding [3].

Comparing the sequences of the IL-12p40 genes of both strains revealed that indeed these subunits differ from each other at two sites, with a Met (C57BL/6) → Thr (SJL/J) substitution at position 169 and a Phe (C57BL/6) → Leu (SJL/J) substitution at position 294 relative to the Methionine startcodon. To test our hypothesis that these changes in amino acids could influence the heterodimer formation, we transfected cells with vectors containing the variant formed of IL-12p40 together with the gene for IL-35. We found that supernatants of cells containing constructs with the SJL/J derived IL-12p40 synthesized significantly more IL-12p70 relative to also secreted free IL-12p40 than did cells containing the C57BL/6 derived chain. Due to the use of constructs the transcriptional and translational regimes for both forms of IL-12p70 are the same and this implies that the difference in synthesis must be attributed to a posttranslational event such as an increased affinity of one subunit for the other. An alternative explanation for the results however could be that the amino acid changes resulted in an impaired secretion of the IL-12p40 free chain from the SJL/J strain. The Met → Thr substitution creates a potential O-glycosylation site which could negatively influence the secretion potential of the IL-12p40 subunit as a consequence of this O-glycosylation. That the glycosylation status can influence secretion was shown by Ha et al [4]. However this possibility was ruled out by determining the concentration of p40 from both strains intracellularly as well as from the supernatant, which revealed an excellent correlation between the amount of intracellular p40 and in the supernatants.

Although proteins are reported to be fully folded and assembled in the endoplasmatic reticulum and O-glycosylation is restricted to the Golgi apparatus (for a review see [5], we investigated the possibility of an influence of O-glycosylation on heterodimer formation by inhibiting the O-glycosylation with the inhibitor Benzyl 2-acetamido-2-deoxy- α -D-galactopyranoside. This unequivocally showed that O-glycosylation is not involved in the increased heterodimer formation as seen in the IL-12p70 synthesis of the SJL/J strain.

Although O-glycosylation does not seem to play a role in the heterodimer formation this does not rule out that it actually took place as Western blotting revealed a higher MW for the free IL-12p40 chains derived from the SJL/J strain. However an alternative explanation for this higher MW might be secondary glycosylation of N-linked sugars. An altered structure in the SJL/J derived chain which promotes heterodimer formation could make the N-glycosylated residues more accessible to secondary glycosylation in the Golgi apparatus. This mechanism is involved in the formation of complex-type N-glycans as opposed to the high-mannose type N-glycans which are thought to be a consequence of inaccessibility of the sugar residues to secondary glycosylation due to protein folding [5].

Mucosal homeostasis is a dynamic process depending on the balance between pro- and anti-inflammatory signals. With a genetic susceptible individual, a defective regulation of immunologic responses could be the cause of a pathogenic response to bacterial, dietary and self-antigens. In the SJL/J mouse strain the same initiating event would inherently give rise to a higher synthesis rate of pro-inflammatory cytokines, skewing the balance of pro- and anti-inflammatory signals to a more pro-inflammatory tendency than in C57BL/6 animals. Thus given a big enough challenge it is wholly conceivable that with SJL/J mice the balance would tip over to a pro-inflammatory route, whereas in the C57BL/6 mouse this process would stay within the bandwidth of homeostasis. In this context it is noteworthy that treatment with bacterial LPS, a strong inducer of IL-12 synthesis, renders genetically resistant C57BL/6 mice as susceptible as SJL/J mice to Theiler's Virus-Induced Demyelinating Disease [6].

The likely involvement of the IL-12p40 gene in TNBS induced colitis prompted us to study its possible role in human Crohns Disease (CD) in the chapters 3 and 4 of this thesis.

Huang et al. [7] described a single nucleotide polymorphism (SNP) (rs3212227) in the human IL-12p40 gene. This SNP is located in the 3'-UTR a region known to be important for posttranscriptional regulation of protein synthesis. Subsequently it was reported that this SNP was associated with type 1 Diabetes Mellitus (IDDM), with severe Asthma and with Psoriasis [8] [9] [10].

In **chapter 3** we investigated whether this SNP is associated with susceptibility to Crohns Disease (CD) by studying the IL-12 secretion by monocytes. We found that rs3212227 was associated with increased secretion of IL-12 p70 by stimulated human monocytes, and that individuals homozygous for the polymorphism were high producers of IL-12p70 in vitro, that

individuals that were heterozygous were intermediate producers, and that non-carriers were low producers. Fascinatingly, and reminiscent of the data reported in chapter 2 we did not find an association between genotype and the ability of monocytes to produce IL-12p40. Thus the 3'-UTR may affect p70 heterodimer formation because it is in linkage disequilibrium with a polymorphism in the coding region that affects heterodimer formation.

In this study we could not establish a significant association of CD with the rs3212227 polymorphism, however this does not exclude a role for the IL-12 gene in the susceptibility to CD. The relative small sample size in combination with the rare frequency of the variant allele may not have been powerful enough to detect such an association. In general, large-scale family-based linkage studies are necessary to establish a role for genes in the susceptibility to multifactorial diseases such as CD.

In **chapter 4** we builded further on these previous observations. Using phenotypic data we screened high and low secretor individuals for the presence of additional polymorphisms in the gene coding for the IL-12p40 subunit. Special emphasis was put on regulatory regions within the promoter region and on the exons where variation in our TNBS colitis studies had revealed the dramatic effect of amino acid changes on the formation of biologically active IL-12 heterodimers. In total 12 individuals that were either high or low secretors of IL-12 p70 were screened using single-strand conformation polymorphism (SSCP). Despite inter-individual differences in IL-12 p70 responses of up to 25-fold, no sequence variation in any of the exons was found.

When we analyzed the 5'-UTR of the IL-12 p40 gene a G→T SNP at position -822 relative to the ATG site was found. We used this SNP together with rs3212227 from the 3'-UTR and a promotor polymorphism described by Morahan et al [9] to extend our genetic studies from chapter 3 to a large group of Caucasian families with at least one affected child (464 trios) and an additional group of 520 healthy Caucasian blood donors from the same geographic area for a case control study. Both case-control analysis as well as the transmission disequilibrium test did not reveal an indication for a direct association with the IL-12p40 gene. Based on these findings we then excluded a direct role for IL-12p40 in disease pathogenesis. However we did not exclude that IL-12p40 variants may play a role in a subgroup of patients or that a more distant polymorphism not included in our SSCP screening could be

involved. This last option was recently confirmed when an association was found between a SNP ~60 kB upstream of the IL-12p40 gene (rs6887695) and CD [11].

That we found no additional informative polymorphisms which would explain in a way analogue to our mouse studies, the strong correlation previously found between the 3'UTR polymorphism and in vitro secretion means that it is either a direct effect of this SNP, or an effect from a more distant polymorphism that was not included in the current analysis, or from other, as yet unknown, mechanism(s).

In the last two chapters we extended on the observations in the mouse with regards to the susceptibility for or resistance to TNBS colitis. As the expression of a substantial number of genes is controlled by interaction with regulatory sequences in the proximity of a gene (so called cis-acting elements) a change in such an element can lead to an altered expression of this gene. This then would give rise to an over- or under representation of its function. This imbalance in its function could then form the underlying cause of disease. Thus assessment of differentially expressed genes in genomic regions associated with disease can help identifying the causative genes for that disease. Micro-array technology is a powerful tool for the identification of hitherto unknown genes by comparing the expression levels of thousands of genes under different conditions.

In **chapter 5** we therefore applied micro-array and bio-informatics in analyzing the pathological processes in experimentally (TNBS) induced colitis, and compared these data with those derived from human IBD (UC and CD). We included in our analysis data from celiac patients, as an example of another inflammatory disease of the intestine, where the primary cause is an excessive cell-mediated immune response to food antigens (gluten). As non-intestinal control we included data derived from rheumatoid arthritis (RA) synovial tissues.

To obtain an overview of the biological processes that are affected in the different diseases, we performed a gene ontology analysis. The more acute character of the mouse TNBS model was clearly reflected by the lack of activation of the specific immune response, which was a prominent feature in UC, CD, and RA. With this analysis we noted a marked difference between celiac disease and the other inflammatory diseases. Whereas in the latter the “immunity and defense” pathways were significantly involved, this was not the case in celiac disease.

As altered transcription levels are the result of altered transcription factor binding, a search for shared transcription factor binding sites in groups of regulated genes can reveal

common regulatory mechanisms. Using such an analysis we found an interesting difference between UC and RA at the one hand and celiac disease at the other. In the acute experimental colitis, and two different chronic human diseases, UC and RA, up-regulated genes were enriched in binding sites for the transcription factor Ets2. As Ets2 is thought to be involved in the increased survival and constitutive production of pro-inflammatory cytokines [12] it may be an important factor in the transition to and sustaining of chronicity of the diseases. In celiac disease however the most significantly enriched binding site in the up-regulated genes was GLI. The Gli family proteins (Gli1-3) are involved in pattern formation, tissue homeostasis and morphogenesis [13] [14]. Prevention of Gli1 binding to target DNA sequences successfully prevented experimental tumor growth [15]. Therefore Gli1 may also be responsible for the increased proliferation rate of crypt epithelial cells leading to crypt hyperplasia, and disappearance of villi, which are the hallmarks of celiac disease.

In **chapter 6** we used micro array technology with a focus on differentially expressed genes in the loci associated with TNBS induced colitis [1]. This way we identified claudin 18 in the *Tnbs1* region on chromosome 9, as a plausible candidate gene involved in susceptibility to colitis. It encodes an important structural protein of the tight junction complex of epithelial cells. Tight junctions seal off the intercellular space between adjacent epithelial cells and regulate passive diffusion of solutes and macromolecules to the underlying lamina propria.

In a previous study on Claudin 18 using tissues from mice and man [16] its presence was reported in lung and stomach but not in the colon. Also in another study specifically addressing expression of claudins along the murine gastrointestinal tract claudin-18 was not reported to be expressed in the colon [17]. We also showed low claudin 18 expression in colon of control mice or non-colitic patients, but found it highly upregulated with inflammation. The failure to detect claudin-18 expression in colon tissue in previous studies must therefore be attributed to the fact that these studies were performed with non-inflamed tissue. Studying the kinetics of expression in the susceptible and resistant mouse strain we found a distinct difference in response time between both strains with regard to a splice variant which lacks its C-terminal cytoplasmic domain, claudin 18A1.2. which would inhibit anchoring to the scaffolding proteins ZO-1, ZO-2, and ZO-3 [18, 19]. The resistant C57BL/6 strain showed a strong upregulation already at 4 hrs after TNBS challenge while at that time in the SJL/J strain expression was still at its basal level. Upregulation in this strain was seen at 8 hrs after chal-

lence. We speculate that this upregulation is part of a repair or defense mechanism whereby upregulation of the variant would lead to loosening of the tight junction. Loosening would enable newly formed cells to migrate into the tissue. Also a regulated opening of the tight junctions forms an integral part of the immune defense system, as immune cells have to migrate across the paracellular space during inflammation. Also dendritic cells have to perform immune surveillance by sticking out protuberances through the paracellular space into the gut lumen [20] which helps the adaptive immune system to sense and react to changes in epithelium homeostasis.

This loosening has to be strictly regulated, as there is the imminent danger of bacterial translocation into the lamina propria. That the resistant C57Bl/6 strain shows rapid downregulation of the truncated form of claudin 18, indicates a fast change in the tight junction topology which could be associated with the prevention of bacterial translocation after the intestinal injury, precluding a better capacity to recover from the colitis.

That claudin-18 could also be involved in human IBD stems from the observation that in UC-patients a significantly higher gene expression was found compared to the control group. Whether this involvement holds true for only UC patients or also CD patients or subgroups needs further studies with larger groups of patients. Our results clearly indicate that claudin-18 plays a role in both an induced animal colitis model as it does in human IBD.

Combining these findings with those reported in chapter 2 one can imagine that in the susceptible SJL/J mouse the sustained IL-12 production, through preferential binding of its subunits, together with putative defects in barrier functions, such as an altered structure of the tight junction based on claudin-18 expression, will lead to chronic inflammation and colitis. That these events are even mechanistically linked can be seen from the fact that IL-12 production leads to enhanced IFN- γ production, which in turn can lead to impairment of colonic epithelial tight junction function [21]

Conclusion

As more and more candidate genes are being discovered in large-scale association studies it becomes clear that these genes can be classified as belonging to either a group that is mainly concerned with immune response genes, and another group that is related to genes involved in the permeability of epithelium. This is not surprising because defects in either function will

result in some sort of disturbance of the intestinal homeostasis. Depending on concomitant factors such as lifestyle, certain microbiota etc this will lead to disease. It is therefore clear that IBD as such is an extremely complex affection involving multiple genes and multiple factors, but eventually all leading to breaching of the intestinal barrier and induction of inflammation. It will therefore be important to further study the mechanisms that underlie the homeostasis of the intestines to better understand the etiology of the disease and to be able to develop better treatment.

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