Carcinogenesis vol.22 no.4 pp.665–671, 2001

Interleukin-10-deficient mice and inflammatory bowel disease associated cancer development

Sanda Sturlan, Georg Oberhuber[1], Britta G.Beinhauer, Brigitte Tichy[1], Sonja Kappel, Jacob Wang[2] and Michael A.Rogy[3]

Department of General Surgery and [1]Department of Clinical Pathology, University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria and [2]Institute for Surgical Research, The National Hospital, University of Oslo, Oslo, Norway

[3]To whom correspondence should be addressed
Email: Michael.Rogy@akh-wien.ac.at

Interleukin-10-deficient mice develop colitis and colorectal cancer similar to the inflammatory bowel disease associated cancer in humans. The aim of this study was to identify possible mutations of oncogenes and tumour suppressor genes involved in tumorigenesis in Interleukin-10 (IL-10)-deficient mice. Twenty colon carcinomas from IL-10-deficient mice were screened for mutations in the *K-ras* and *p53* genes by 'cold' single-strand-conformation polymorphism. Immunohistochemical staining was performed to detect mutations in the proteins P53, APC and MSH2, and the transforming growth factor β type II receptor. Microsatellite instability was analysed at eight chromosomal loci and plasma levels of transforming growth factor β1 (TGF-β1) were also measured. At 9 weeks, 14% of the animals developed colorectal cancer, and at 10–31 weeks the incidence of carcinoma was 65%. No mutations were detected in the analysed oncogene and tumour suppressor genes. Plasma TGF-β1 levels in IL-10-deficient mice 10–31 weeks old were higher than in wild-type littermates e.g. 45.7 ± 4.6 ng/ml versus 19.8 ± 4.5 ng/ml ($P < 0.01$). No alterations in *K-ras*, *p53*, *Apc* and *Msh2* genes suggests that other genes are involved in the development of these tumours. Elevated TGF-β1 plasma levels correspond to the high incidence of dysplasia and cancer. Normal expression of the TGF-β II receptors hints at genetic alterations in other members of the TGF-β receptor signal transduction pathway.

Introduction

Interleukin-10-deficient mice, maintained under specific-pathogen free (SPF) conditions, spontaneously develop chronic enterocolitis, a condition phenotypically similar to chronic inflammatory bowel disease (IBD) in humans (1), at 6 weeks of age. Intensive study of these animals should lead to a better understanding of the complex mechanisms involved in the initiation and progression of IBD. Recently it has been shown that 6-month-old IL-10-deficient mice have a high incidence of adenocarcinomas, which is mostly dependent on their genetic background (2). We have observed that C57BL/6 mice are not resistant to colorectal adenocarcinoma. Given these findings, this murine IBD model may provide an excellent model for studying the patho-mechanism of IBD-associated carcinomas.

Patients suffering from IBD have an increased risk of colorectal carcinoma. In contrast to sporadic colorectal cancers, which develop through the 'adenomatous polyps sequence', IBD-associated carcinomas develop through the 'dysplasia sequence'. Although sporadic colorectal carcinoma as well as cancer from IBD are hypothesized to arise by a multi-step process involving genetic instability and clonal expansion, it seems that the mechanisms differ. The genetic alterations involved in the initiation and development of sporadic and familial colorectal carcinomas are mostly known. The most frequent genetic alterations in sporadic colorectal carcinoma are mutations of the *P53*, *APC* and *DCC* tumour suppressor genes, as well as mutations of the *KRAS* proto-oncogene occurring either early or late in carcinogenesis. Familial colorectal cancer susceptibility is associated with germline mutations of specific genes. Germline mutations of the *APC* gene are responsible for the development of familial adenomatous polyposis (FAP), whereas the hereditary non-polyposis colorectal cancer (HNPCC) syndrome is caused by germline mutations of the mismatch repair (MMR) genes, *hMLH1*, *hPMS1*, *hPMS2* and *hMSH2*.

For a better understanding of carcinogenesis, mice models with targeted mutations in tumour suppressor or oncogenes were generated. Although genetic alterations in mice and humans do not always result in the same type of tumours, the basic molecular mechanisms are often relevant for both species. IL-10-deficient mice develop colorectal carcinoma through the dysplasia sequence, which is histologically similar to IBD-associated cancers in humans. The purpose of this study was firstly to identify the possible mutations of oncogenes and tumour suppressor genes involved in the induction and progression of tumours in IL-10-deficient mice, and secondly to look for similarities in genetic alterations involved in IBD-associated carcinomas between humans and this murine model.

Materials and methods

Animals

All animal experiments were investigated and approved by both the Animal Ethic Committee of the University and the Ministry of Science prior to the studies (GZ 66.009/22). Interleukin 10 (–/–) mice generated on a C57BL/6 background (3) and wild-type (wt) littermate controls were purchased from B & K Universal (Hull, UK). Animals were kept under specific-pathogen free (SPF) conditions and fed a standard diet and water *ad libitum*. All mice received human care in accordance with the National Institutes of Health guidelines and the legal requirements in Austria.

Examination of mice

The entire small and large intestine were examined in 48 IL-10 (–/–) and 14 IL-10 (+/+) mice between 9 and 31 weeks of age. Moribund IL-10 (–/–) mice and age-matched wild-type mice were killed by cervical dislocation and then autopsied. The small and large intestines were fixed in PBS + 4.5% formaldehyde before embedding in paraffin blocks. For histological analysis, 5 µm thick sections were cut and stained with hematoxylin and eosin (H&E).

Abbreviations: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colorectal cancer; IBD, inflammatory bowel disease; MMR, mismatch repair; PCR, polymerase chain reaction; SPF, specific-pathogen free; SSCP, single-strand conformation polymorphism; TGF-β, transforming growth factor β.

© Oxford University Press

Table I. Primer pairs used in this study

Location	Amplimer size (bp)	Primer name	Primer sequence
p53 exon 6	255	p53 intron 5S	5′-CCTCAACACCGCCTGTGGGGTTAG
		p53 intron 6AS	5′-GAAAGTCAACATCAGTCTAGGCTG
p53 exon 7	234	MP7-56F	5′-CAGGTCACCTGTAGTGAGGTAGGG
		MP7-56R	5′-TGGAACAGAAACAGGCAGAAGCTGG
p53 exon 8	269	p53 intron 7S	5′-TTTACACACAGTCAGGATGGGGCC
		p53 intron 8AS	5′-AAGAGGTGACTTTGGGGTGAAGCTC
K-ras codon 12–13	102	K12S (4-11)	5′-TATAAACTTGTGGTGGTTGGAGCT

Histopathologically, dysplasia was classified as either low-grade or high-grade according to Riddell *et al*. (4).

DNA extraction

The tumour tissue area and the adjacent area of normal intestinal tissue were separately dissected under the microscope and collected in Eppendorf tubes. The genomic DNA was purified from these specimens using proteinase K lysis mix (50 mM Tris–HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, 500 µg/ml proteinase K). In brief, the sections were deparaffinized, rehydrated and treated with proteinase K lysis buffer overnight at 55°C, followed by centrifugation at 14 000 *g* for 20 min. The DNA from the supernatant was precipitated with 100% ethanol, washed with 75% ethanol, dried and resuspended in dH_2O. Tail DNA from wild-type mice extracted by proteinase K digestion and phenol–chloroform was used as a control tissue DNA.

Detection of single strand conformation polymorphism

PCR-based non-radioactive single strand conformation polymorphism ('cold' SSCP) was used to screen for the presence of *p53* and *K-ras* mutations in tumours. Table I shows the sequence of four primers used in our study. Genomic DNA (100 ng) was used as a template in a reaction volume of 25 µl containing 20 pmol each primer, 50 µM each dNTP and 1 U Golden *Taq* DNA polymerase (Perkin Elmer, Foster City, CA). PCR reactions were carried out in a Perkin Elmer Thermal Cycler 480 for 45 cycles. Annealing temperatures for each primer set were optimized in pilot studies before processing experimental samples. The PCR products were purified from primer dimers using the Concert Rapid PCR Purification System (Gibco BRL, Rockville, MD) and analysed for yield and purity by electrophoresis on 10% polyacrylamide mini gels (Novex, San Diego, CA). A 10 µl aliquot of purified PCR product mixed with loading buffer was heat denaturated by boiling for 5 min, placed immediately on ice and loaded on the gel. Either 20% (for exon 7 of *p53* and codon 12–13 of *K-ras*) or 4–20% gradient (for exon 6 and 8 of *p53*) pre-cast polyacrylamide Tris/borate/EDTA (TBE) gels were used (Novex, San Diego, CA). Optimal temperatures and other electrophoretic conditions were empirically predetermined according to Hongyo *et al*. (5). Electrophoresis was run at constant voltage (300 V) on a Thermo Flow Electrophoresis Temperature Control (ETC) System (Novex, San Diego, CA). Buffer temperature, gel type and fragment size determined the running time. Circulating TBE buffer was maintained at 8°C for exon 7 (*p53*) and codon 12–13 (*K-ras*), at 10°C for exon 6 (*p53*) and 24°C for exon 8 (*p53*). The gels were stained with Syber Green II (Novex, San Diego, CA) and photographed under UV light.

Sequence analysis

SSCP data were verified by sequencing. Genomic PCR products of normal and tumour specimens were used as templates for direct sequencing. Prior to sequencing, the PCR products of *p53* (exons 6–8) and *K-ras* (exon 1, codon 12–13) genes were purified from single-stranded DNA residues. The sequencing reaction mixture consisted of 2 µl purified PCR product, 2 pmol primer (sense or antisense) and 2 µl Terminator Ready Reaction Mix (Perkin Elmer Applied Biosystems), in a total volume of 10 µl. Sequencing was performed in a T1 thermocycler (Biometra, Goettingen, Germany) for 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The product was purified by precipitation in 75% isopropanol and analysed in a ABI Prism 310 Genetic Analyser (Perkin Elmer, Foster City, CA).

Microsatellite instability in colorectal tumours

Tumour tissue and adjacent normal colonic tissue were analysed for microsatellite instability at eight chromosomal loci: *D17Mit123*, *D14Mit15*, *D10Mit2*, *D7Mit91*, *D6Mit59*, *D4Mit27*, *D3Mit203* and *D1Mit36*. Microsatellite primer sequences were obtained from the Whitehead Institute for Genome Research (Cambridge, MA). Five of these amplify sequences containing CA repeats, and the other three (*D4Mit27*, *D3Mit203*, *D1Mit36)* amplify loci containing GT repeats. PCR products (12 µl) were mixed with 5 µl loading buffer and loaded onto a 0.8 mm thick 24×18 cm, 7% non-denaturing polyacrylamide gel. Electrophoresis was performed in 1× TBE buffer at 850 V/h at room temperature. DNA fragments were stained with Syber Green I (Novex, San Diego, CA) and visualized by UV under detector at 250 nm.

Immunohistochemistry

Immunohistochemical staining was performed on 5 µm thick, formalin-fixed, paraffin embedded tissue sections. Sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in water. Antigen retrieval was accomplished by microwave irradiation. After being blocked in 10% normal horse/rabbit/goat serum for 15 min at room temperature, the serial sections were incubated with one of the following primary antibodies, respectively: (i) mouse anti-P53 monoclonal Ab (Ab-3, clone Pab 240; Calbiochem, Cambridge, CA), diluted 1:500; (ii) sheep anti-P53 polyclonal Ab (Ab-7; Calbiochem), diluted 1:10; (iii) rabbit anti-P53 polyclonal Ab (Signet Laboratories, Dedham, MA), diluted 1:10 for 1 h at room temperature. After brief rinsing, sections were treated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, rinsed and incubated with avidin–biotin complex (Vectastain ABC Peroxidase kit; Vector Laboratories), for 45 min at room temperature. For visualization of rabbit anti-P53 polyclonal antibodies, incubation with goat anti-rabbit secondary antibodies was followed by incubation with Strep-AB Complex/HRP (DAKO A/S Glostrup, Denmark), for 30 min at room temperature. The peroxidase activity was visualized by application of the chromogen 3,3′-diaminobenzidine. The sections were then counterstained with hematoxylin, dehydrated, cleared and mounted.

For the detection of the APC protein we used APC (N-15), a rabbit polyclonal antibody raised against the N-terminus of the APC protein (Santa Cruz Biotechnology, Santa Cruz, CA), and APC (C-20), a rabbit polyclonal antibody raised against the C-terminus of the APC protein (Santa Cruz Biotechnology). Following removal of paraffin in xylene, tissue rehydration was performed employing descending grades of alcohol (100–50%). The endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2 in methanol for 30 min, followed by rinsing with running tap water. The antigen retrieval was accomplished by treatment with 0.1% protease type XIV (Sigma, St Louis, MO) for 10 min at 37°C. The reaction was stopped by washing twice in cold Tris–HCl buffer 7.4. Non-specific antigens were blocked by pre-incubation with 10% normal goat serum for 15 min at room temperature. The primary antibodies N-15 and C-20 were diluted 1:250 and 1:50, respectively, in 0.5 M Tris–HCl buffer pH 7.5, containing 2% BSA and applied for 1 h at room temperature. After brief washing, sections were treated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min at room temperature. Additional washing was followed by incubation with the avidin–biotin complex for 45 min at room temperature. The reaction was developed using the chromogen 3,3′-diaminobenzidine.

Detection of MSH2 proteins was performed with mouse monoclonal antibody (clone FE11; Calbiochem) diluted 1:5, following the procedure described for Ab-3 and Ab-7 in P53 protein detection.

For the detection of the TGF-β1 II receptor we used TGF-β1 RII (C16), a rabbit polyclonal antibody raised against a peptide mapping within the C-terminal domain of the TGF-β1 II receptor (Santa Cruz Biotechnology). The endogenous peroxidase was blocked by incubation with 0.6% H_2O_2 in methanol for 30 min, followed by rinsing with tap water. After being blocked in 10% normal goat serum for 15 min at room temperature, the sections were incubated overnight at 4°C with the primary antibody diluted 1:150. After brief washing, sections were treated with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 min at room temperature, washed and incubated with Strep-AB Complex/HRP (DAKO A/S Glostrup, Denmark) for 30 min at room temperature. The immunoreaction was visualized using 3,3′-diaminobenzidine.

Analysis of TGF-β1 levels

Immediately prior to being killed, mice were anaesthesized with phenobarbital (35 mg/kg body weight) and bled from the heart. Plasma with a low platelet

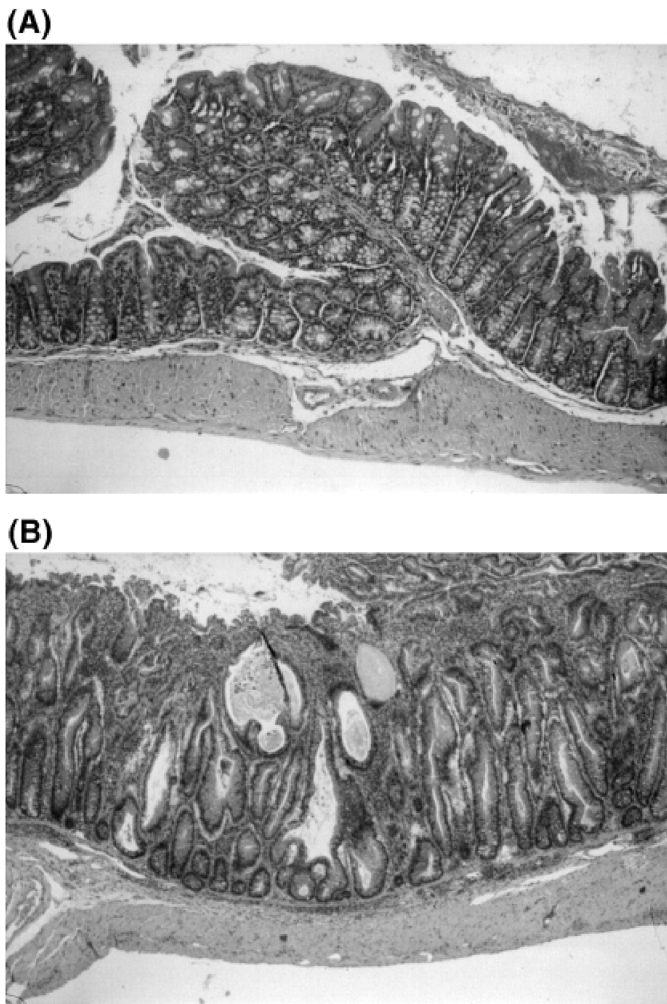

Fig. 1. (**A**) Normal appearing colonic mucosa in IL-10-deficient mice. H&E. Original magnification, ×200. (**B**) High grade dysplasia of colonic mucosa. Please note enlarged and irregular colonic crypts. The nuclei atypia. H&E. Original magnification, ×200.

count was obtained according to the manufacturer and stored at –80°C. Plasma levels of TGF-β1 were measured by enzyme-linked immunosorbent assay, using a Human TGF-β1 Immunoassay kit (R&D System, Minneapolis, MN). The sensitivity of the assay was $<$7 pg/ml.

All experiments were statistically analysed by the JMP program (Macintosh version). Evaluation of changes in cytokine concentrations was done by ANOVA and the Newman–Keuls multiple range test. All significance limits were determined at the 99 percent confidence level.

Results

Development of colorectal dysplasias and carcinomas in IL-10-deficient mice

Histological analysis showed that in contrast to wild-type littermates at the age of 9 weeks, all IL-10 (–/–) mice developed colitis. We have also observed that the small intestine was not affected, and there was no histological difference of the small intestine between IL-10 (–/–) and IL-10 (+/+) animals. At 9 weeks, 13 out of 22 animals (59%) suffering from colitis developed high grade dysplasia (Figure 1) and 3 animals (14%) were found to have adenocarcinomas. In 10–31-week-old mice, the incidence of colorectal carcinomas was much higher, e.g. 17 out of 26 animals (65%), suggesting rapid progression of dysplasias to carcinomas (Table II). Some tumours infiltrated the muscularis propria (Figure 2) but no metastasis in mesenteric lymph nodes and liver were observed at this age.

Analysis of K-ras and p53 mutations

Twenty colorectal tumours were screened for the presence of activating mutations in codon 12–13, using PCR-based cold SSCP method and sequencing. Surprisingly, there were no *K-ras* point mutations in any of the tumours examined (Figure 3).

To analyse whether the overexpression of the P53 protein correlates with the initiation and progression of colorectal tumours in IL-10 (–/–) mice, we used one type of monoclonal antibody and two types of anti-P53 polyclonal antibodies. None of the examined tumours showed positive staining, indicating that there was no overexpression of P53 protein.

Among the variety of mutations which inactivate *p53*, point mutations at exons 6–8 tend to predominate. Each of the colorectal DNA samples were examined by cold SSCP analysis of exons 6–8 of the *p53* gene. There was no altered electrophoretic mobility in any of the examined PCR products (Figure 4) and sequencing revealed no mutations.

APC mutation

The status of the APC protein in colorectal tumours in IL-10 (–/–) mice was analysed using monoclonal antibodies specific for the N- and C-terminus of APC. Immunohistochemical staining showed no difference between tumour and adjacent normal colonic tissue (Figure 5).

Microsatellite instability

To analyse the stability of microsatellite sequences from both tumours and the surrounding normal colonic tissue, eight different sets of primers were used. As shown in Figure 6, none of the samples show any change in dinucleotide repeat sequences, indicating no microsatellite instability in tumours developed in IL-10 (–/–) mice.

MSH2 expression in colorectal carcinomas

To determine MSH2 expression, all tumours were subjected to immunohistochemical analysis using a mouse anti-MSH2 polyclonal antibody. All examined specimens showed nuclear staining both in the tumour and the surrounding normal tissue.

Plasma TGF-β1 levels in IL-10-deficient mice

Plasma TGF-β1 levels in IL-10 (–/–) mice of age 10–31 weeks were higher (45.7 $\pm$ 4.6 ng/ml) than in wild-type littermates (19.8 $\pm$ 4.5 ng/ml), $P < 0.01$ (Figure 7). IL-10 (–/–) mice of age 10–31 weeks showed 8-fold higher TGF-β1 plasma concentrations (45.7 $\pm$ 4.6 ng/ml) than those aged 5–7 weeks (5.5 $\pm$ 1.6 ng/ml), $P < 0.01$. These dramatic increases are associated with the incidence of dysplasias and cancers in examined mice.

Expression of the TGF-β II receptor

To examine the expression of the TGF-β II receptor in tumour tissue, all tumours were analysed with monoclonal antibodies specific for the C-terminus of the TGF-β II receptor. Immunohistochemical staining showed no difference between tumour and adjacent normal colonic tissue.

Discussion

In our study we have observed that IL-10-deficient mice, although kept under SPF conditions, suffer from colitis from 6 weeks of age. They develop high grade dysplasia at 9 weeks and finally cancer. According to our data, it seems that the

Table II. Results of histological analysis of large intestines of IL-10-deficient mice

Mice genotype	Age (weeks)	*n*	Colitis	Hyperplasia	LG dysplasia	HG dysplasia	Cancer
IL 10 (−/−)	9	22	100%	100%	18%	59%	14%
	10–31	26	100%	100%	0	31%	65%
IL 10 (+/+)	9	6	0	0	0	0	0
	10–31	8	0	0	0	0	0

n, Number of animals per group; LG, low grade; HG, high grade.

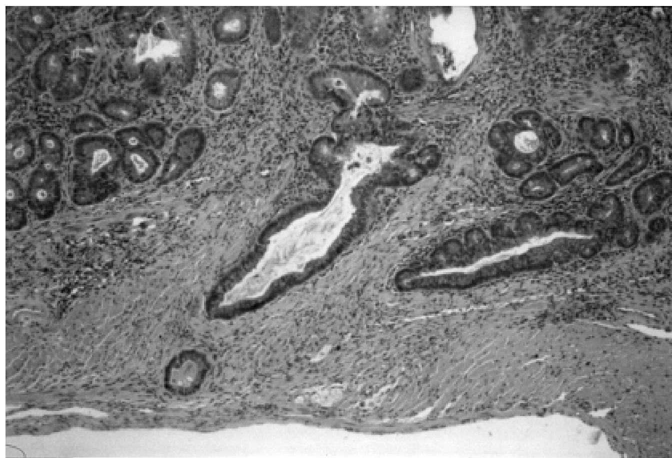

Fig. 2. Well differentiated adenocarcinoma invading into the muscularis propria in IL-10-deficient mice. H&E. Original magnification, ×250.

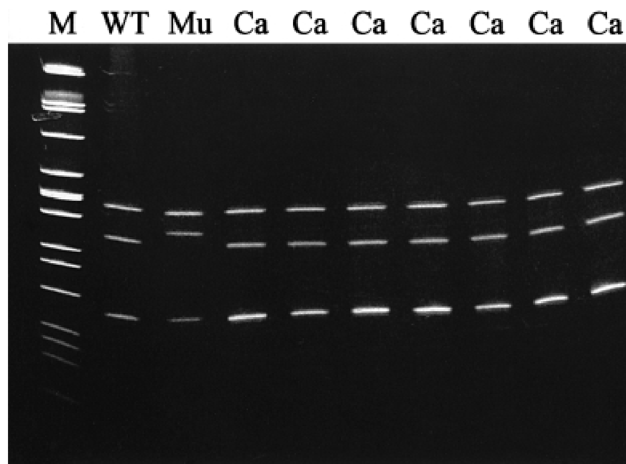

Fig. 3. PCR–SSCP analysis of DNA from the *K-ras* exon 1 (codon 12–13). M, DNA molecular weight marker; WT, wild type; Mu, mutated; Ca, cancer. There is no altered electrophoretic mobility in any of the tumour samples.

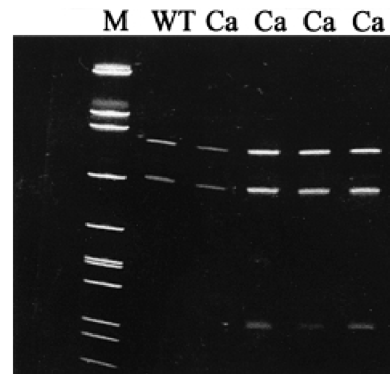

Fig. 4. PCR–SSCP analysis of DNA from *p53* exon 6. M, DNA molecular weight marker; WT, wild type; Ca, cancer.

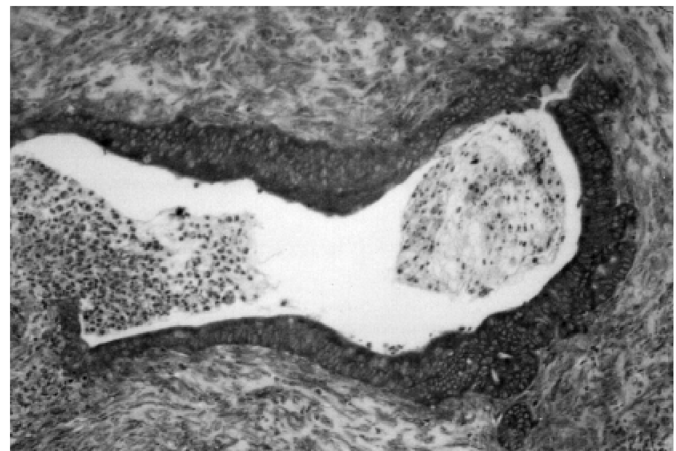

Fig. 5. Immunohistochemical localization (brown staining) of APC in colonic cancer of IL-10-deficient mice. Immunoperoxidase. Original magnification, ×300.

occurrence of dysplasia and cancer in IL-10-deficient mice is not dependent on the genetic background, as previously believed (2). The relatively short period between development of colitis with dysplasia and carcinoma (6 versus 9 weeks) in these animals, makes it hardly possible to follow up the chronic inflammation for a long period of time. Also, this event may result in these mice being unresponsive to different types of therapy against chronic inflammation. From our data we can confirm that IL-10-deficient mice may be considered as an appropriate animal model for the study of IBD; however, only for a short period of time. After that, the possible development of dysplasia and cancer should be kept in mind in any kind of experiments using this model.

We have also observed the high similarities in the histopathological pattern of colorectal cancers between IL-10-deficient mice and IBD-associated carcinomas in humans. Both of them develop from flat dysplastic epithelium rather than from adenomas. According to these findings, it might be possible that the genetic alterations involved in tumorigenesis are similar too. To gain further insight into the molecular basis of carcinogenesis in these mice we have analysed 20 colorectal tumours for mutations in the *KRAS, p53*, *APC*, *MSH2* and *TGF*-β receptor *II* genes and microsatellite instability; all of them known to have an important role in the initiation and progression of familial, sporadic and IBD-associated tumours.

Mutations of the K-Ras oncoprotein occur in 40–60% of sporadic colorectal cancers. The single point mutations occurring at codons 12 and 13 or 61 are an early event in the development of sporadic colorectal carcinomas. However, the role of *KRAS* in IBD-associated carcinomas is not clearly

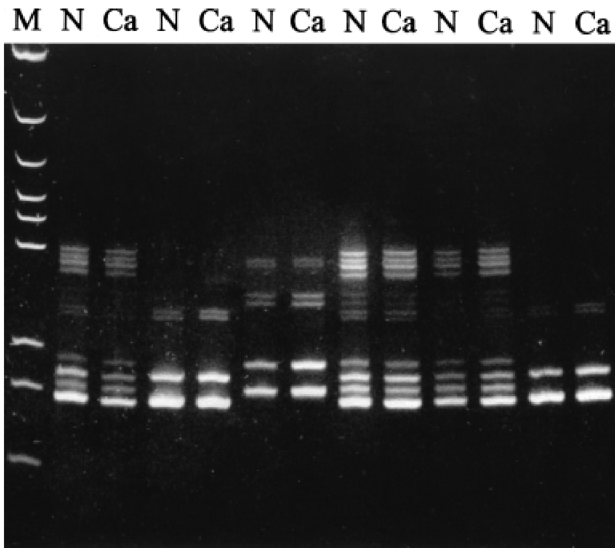

Fig. 6. Analysis of microsatellite instability in colorectal cancers from IL-10-deficient mice. Test with *D1Mit36* marker. M, DNA molecular weight marker; Ca, cancer; N, adjacent normal colonic tissue. No microsatellite instability was observed in any of the tumour samples.

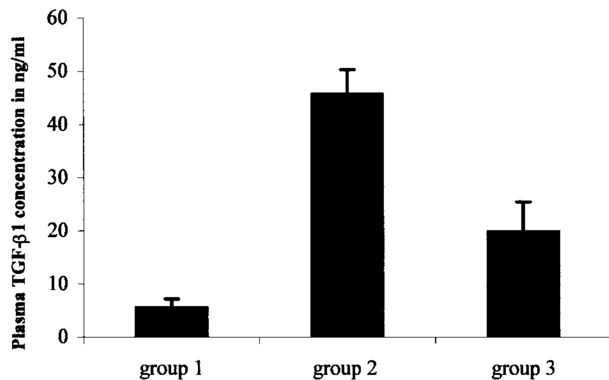

Fig. 7. Plasma TGF-β1 levels in the following groups: group 1, IL-10-deficient mice 5–7 weeks of age; group 2, IL-10-deficient mice 10–31 weeks of age; group 3, wild type mice 10–31 weeks of age. Plasma TGF-β1 was measured by enzyme-linked immunosorbent assay. Bars represent mean values.

defined. Previous studies have shown either a high (up to 40%) (6), or rather low (5–8%) incidence of *KRAS* mutations (7). In order to determine the role of *K-ras* mutations in the development of colorectal cancers in this model, we used the SSCP method to detect single point mutations of codons 12 and 13; reported as the most frequently mutated codons (8). No mutations were detected in the *K-ras* oncogene exon 1 (codons 12 and 13). In previous reports, *K-ras* mutations were present at a high percentage in chemically induced prostate adenocarcinoma, in neurogenic sarcomas in rats (9) and mouse lung tumours (10). Our data are in accordance with the results of Smits *et al.* (11) who showed a lack of *ras* mutations in intestinal tumours from *Apc*1638N, a mouse model for *Apc*-driven carcinogenesis. Similar data were shown in chemically induced mouse stomach tumours (12). There seems to be evidence that gastrointestinal cancers in mice are not driven by *ras* mutations. However, to confirm this hypothesis other members of the *ras* family, namely *Hras* and *Nras*, have to be analysed in our model as well. Another reason for the lack of *K-ras* mutations might be due to the mice strain. C57BL/6 mice, used in our study, are considered as strain 'resistant' to *K-ras* mutations (11).

Inactivation of the *p53* tumour suppressor gene is one of the most frequent mutations occurring in 70% of sporadic colorectal carcinomas and about 50% of IBD-associated colorectal carcinomas in humans. Genetic alterations of *p53* in sporadic colorectal carcinoma are reported as a late event, whereas in the case of IBD-associated carcinomas there is conflicting evidence as to whether *p53* mutations are early (13) or late event (14). The inactivation of *p53* fits in with the 'two hits' hypothesis which includes allelic loss in the *p53* gene followed by the missense mutation in the other allele. The oncogenic potential of a *p53* mutation is not only due to the tumour suppressive function but also to the dominant negative effect which is caused by the dimerization of mutant *p53* with the wild-type protein. This drives the wild-type into the mutant conformation. Both events result in nuclear or cytoplasmatic accumulation of mutated protein, which can be detected by immunohistochemistry. Although we examined the most frequently mutated exons (exons 6–8), no mutations were detected by the PCR-based SSCP method. In order to avoid the possibility that mutations of other non-examined exons would lead to inactivation of *p53*, we used the immunohistochemical method to visualize mutated protein. This method did not detect any mutations of *p53* in the examined tumours either. A possible explanation for this result might be the fact that *p53* null or heterozygous mice ($p53^{+/-}$) develop soft tissue sarcomas, osteosarcomas and lymphomas rather than carcinomas (15). These findings, together with the previous reports of the rare occurrence of *p53* mutations in chemically-induced lung and liver tumours, as well as intestinal tumours of *Apc* null mice, lead to the suggestion that *p53* mutations are not involved in malignant transformation of epithelial cells in mice. Also, inactivation of *p53* may occur in the late stage of colorectal cancers developing in IL-10-deficient mice, thus being present in a small number of tumour cells and barely detectable by the methods used in our study. Another possible reason might be that mutations at other exons do not result in accumulation of the protein. However, it seems very unlikely that more detailed and extensive investigations of the other exons would result in the detection of *p53* mutations.

Germline mutations of *APC* occur in 90% of adenomatous polyps, developed through FAP. Somatic mutations of the *APC* gene are also found in 60% of sporadic colon cancers as well as intestinal tumours from patients suffering from hereditary non-polyposis colorectal cancer (HNPCC). In general, the frequency of the *APC* gene attenuation is similar in sporadic colorectal adenomas and cancers, indicating that the *APC* mutation represents an early event in the development of sporadic and familial colorectal cancers. The incidence of *APC* mutations in IBD-associated cancer is much lower (6%) than in sporadic tumours (16), showing that this genetic change is not involved in the initiation of IBD-associated carcinogenesis but more possibly occurs in the later stages. Most of the identified *APC* mutations (>95%), both germ-line and somatic mutation result in a truncated APC protein (17). In our study, we have used immunohistochemistry in order to detect this truncated APC protein. The results also show that *APC* mutations are not involved in the initiation of cancer, which is in accordance with previous reports concerning mutations in carcinomas developed through the dysplasia sequence (16).

Germline mutations of mismatch repair genes (MMR),

hMLH1, *hPMS1*, *hPMS2* and *hMSH2*, are responsible for HNPCC (18). In tumours, the wild-type allele is lost through allelic loss or mutational inactivation. Somatic mutations (including allele loss) inactivating both mismatch repair alleles have also been reported in 20% of sporadic colorectal cancers (19). Previous studies showed that tumours with the MMR gene mutation display genetic instability characterized by alteration in microsatellites. The microsatellite instability phenotype was defined in neoplastic (20) and non-neoplastic tissue from patients with ulcerative colitis (21). Although at present, it is not clear whether this phenotype is caused by genetic alterations of MMR genes (22), or by chronic inflammation, which can damage DNA over a long period of time, thus exceeding the capacity of the DNA repair machinery and resulting in gene mutations causing genetic instability (23).

In our study, none of the tumours showed microsatellite instability at any of the loci examined. Immunohistochemical examination did not show a truncation mutation of the MSH2 protein either, although it was shown that MSH2-deficient mice develop intestinal tumours. A possible reason for the lack of microsatellite instability might be that in IL-10-deficient mice the genomic instability during tumorigenesis is due to chromosomal instability caused by chronic inflammation rather than to microsatellite instability.

Increased plasma levels of TGF-β1 corresponding to the age and occurrence of dysplasia and colorectal cancers in IL-10-deficient mice is consistent with previous data, which showed an increase of plasma TGF-β1 levels in patients suffering from colorectal cancer (24). The TGF-β family consists of multifunctional polypeptides known to regulate cell proliferation, cell differentiation, adhesion and migration. TGF-β appears to be one of the most potent negative growth regulators for epithelial cells, especially for the intestinal epithelium. It has been suggested that TGF-β is a physiological modulator of normal intestinal epithelial growth, probably acting in an autocrine fashion. The role of TGF-β in tumour development seems to be dual, and dependent on the stage of the tumour. Thus TGF-β may act as a tumour suppressor in the earliest stages of carcinogenesis, but it can stimulate invasiveness later on. This may be related to the loss of tumour responsiveness on the growth inhibitory effect of TGF-β and at the same time to the TGF-β induced angiogenesis, and local and systemic immunosuppression. It has been shown that TGF-β has an important immunomodulatory role acting either as an initiator or suppressor of the immune response. One of the anti-inflammatory roles of TGF-β is the inhibition of the respiratory burst of macrophages and the suppression of TNF-α and other pro-inflammatory cytokines. The increased level of plasma TGF-β1 in IL-10-deficient mice may be one way the animal tried to 'compensate' for the missing anti-inflammatory cytokine IL-10. The fact that the overexpression of TGF-β1 in plasma occurred at the same age in mice as the occurrence of dysplasia and cancers, led us to the conclusion that the elevated level was also an attempt to inhibit these events. The importance of TGF-β tumour suppressor activity was demonstrated by Engle *et al*. (25) who showed a high incidence of inflammation-associated cancer in *Tgf*-$\beta^{-/-}$ $Rag^{-/-}$ mice. Similar to our findings, those cancers also did not show microsatellite instability or mutations of the *Apc* gene, suggesting that TGF-β has no influence on genetic stability. Moreover, the authors showed that the tumour-suppressor activity of TGF-β is neither due to inhibition of cell proliferation nor suppression of inflammation, but rather due to an ability to maintain the epithelial tissue organization. However, our results show that despite elevated TGF-β levels, 31% of IL-10 deficient mice develop dysplasia and 65% cancer. Our findings are in line with the fact that 20% of $Rag^{-/-}$ *Tgf*-$\beta^{+/+}$ mice develop cancer as well. This demonstrates that normal as well as elevated TGF-β levels are not enough to prevent dysplasia as the consequence of chronic inflammatory stress in these animals.

To examine whether the unresponsiveness of tumours to elevated levels of TGF-β is due to decreased expression of the TGF-β II receptor, all tumour specimens were analysed immunohistochemically. Immunohistochemical results showed that, in IL-10-deficient mice, downregulation and truncation of the TGF-β II receptor is not the reason for the resistance of tumours to the growth-inhibitory effects of TGF-β1. Previous reports have also shown that the loss of TGF-β receptors is not an exclusive event causing tumour proliferation (26). According to this we believe that in our model the tumour development involves a receptor downstream mechanism; including alterations of one of the members in the signal transduction pathway, activated through the TGF-β family receptors. Some of the most interesting candidates for future analysis are the members of the SMAD family, SMAD2, SMAD3 and SMAD4. It has been suggested that *SMAD2*, located on chromosome 18q21, is one of the tumour suppressor genes mutated in human sporadic colorectal carcinomas (27). Mutations in the *SMAD4* (*DPC4*) gene, located on the same chromosome, have also been found in colorectal carcinomas (28). Germline mutation of *SMAD4* has also been described in familial juvenile polyposis, an autosomal dominant syndrome that predisposes the patient/host to hamartomatous polyps and gastrointestinal cancers (29). In addition, heterozygous inactivation of *Smad4* in mice caused gastrointestinal polyposis similar to human juvenile polyps (30). Mutations in *Smad3* are not associated with human cancer. Zhu *et al*. (31) showed that SMAD3-deficient mice develop colorectal carcinoma. Interestingly, both SMAD3 and IL-10-deficient mice develop colorectal cancers, but without neoplastic lesions in the small intestine. In addition, the lack of TGF-β II receptor downregulation and truncation in IL-10-deficient mice may suggest also a possible involvement of a pathway independent of TGF-β1, which may affect the same cellular process affected by TGF-β.

Despite the high similarities between the histopathological pattern of colorectal cancers in IL-10-deficient mice and IBD-associated carcinomas, no correlation in genetic alterations has led us to the conclusion that IL-10-deficient mice are not an appropriate model for investigating IBD-associated carcinogenesis. However, the finding that there are no mutations of the known oncogenes or suppressor genes in IL-10-deficient mice also tells us that some other undiscovered genes are involved in the development of these tumours. This makes IL-10-deficient mice even more of an interesting model for novel insight into tumorigenesis. Looking for mutations of new genes downstream in the TGF-β receptor cascade will give us a better understanding of mechanisms involved in the pathogenesis of neoplastic lesions.

Acknowledgements

This work was supported by grants P13550-med and P11135-med from the FWF-Erwin Schrödinger Stiftung, and in part by the Surgical Research Institute, National Hospital Oslo, Norway.

References

1. Rennick,D.M., Fort,M.M. and Davidson,N.J. (1997) Studies with IL-10 –/– mice: an overview. *J. Leukoc. Biol*., **61**, 389–396.
2. Berg,D.J., Davidson,N., Kühn,R., Müller,W., Menon,S., Holland,G., Thompson-Snipes,L., Leach,M.W. and Rennick,D. (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4+TH1-like responses. *J. Clin. Invest*., **98**, 1010–1020.
3. Kühn,R., Löhler,J., Rennick,D., Rajewsky,K. and Müller,W. (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, **75**, 263–274.
4. Riddell,R.H., Goldman,H., Ransohoff,D.F., Appelman,H.D., Fenoglio,C.M., Haggitt,R.C., Ahren,C., Correa,P., Hamilton,S.R., Morson,B.C., Sommers,S.C. and Yardley,J.H. (1983) Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum. Pathol.*, **14**, 931–968.
5. Hongyo,T., Buzard,G.S., Calvert,R.J. and Weghorst,C.M. (1993) 'Cold SSCP': a simple, rapid and non radioactive method for optimized single strand conformation polymorphism analyses. *Nucleic Acids Res*., **16**, 3637–3642.
6. Holzmann,K., Klump,B., Borchard,F., Hsieh,C.J., Kühn A., Gaco,V., Gregor,M. and Porschen,R. (1998) Comparative analysis of histology, DNA content, *p53* and Ki-*ras* mutations in colectomy specimens with long-standing ulcerative colitis. *Int. J. Cancer*, **76**, 1–6.
7. Burmer,G.C., Levine,D.S., Kulander,B.G., Haggitt,R.C., Rubin,C.E. and Rabinovitch,P.S. (1990) C-Ki-*ras* mutations in chronic ulcerative colitis and sporadic colon carcinoma. *Gastroenterology*, **99**, 416–420.
8. Devereux,T.R., Anderson,M.W. and Belinsky,S.A. (1991) Role of *ras* protooncogene activation in the formation of spontaneous and nitrosamine-induced lung tumors in the resistant C3H mouse. *Carcinogenesis*, **2**, 299–303.
9. Sukumar,S., Armstrong,B., Bruyntjes,J.P., Leeav,I. and Bosland,M.C. (1991) Frequent activation of the Ki-*ras* oncogene at codon 12 in *N*-methyl-*N*-nitrosourea-induced rat prostate adenocarcinomas and neurogenic sarcomas. *Mol. Carcinog*., **4**, 362–368.
10. You,M., Candrian,U., Maronpot,R.R., Stoner,G.D. and Anderson,M.W. (1989) Activation of the K-*ras* protooncogene in spontaneously occurring and chemically induced lung tumors of the strain A mouse. *Proc. Natl Acad. Sci. USA*, **86**, 3070–3074.
11. Smits,R,. Kartheuser,A., Jagmohan-Changur,S. *et al*. (1997) Loss of *APC* and the entire chromosome 18 but absence of mutations at the *ras* and *Tp 53* genes in intestinal tumors from *APC*1638N, a mouse model for *APC*-driven carcinogenesis. *Carcinogenesis*, **18**, 321–327.
12. Furihata,C., Tatematsu,M., Saito,M., Ishida,S., Nakanishi,H., Inada,K., Tei,H., Hattori,M., Ito,T. and Sakaki,Y. (1997) Rare occurrence of *ras* and *p53* gene mutations in mouse stomach tumors induced by *N*-methyl-*N*-nitrosourea. *Jpn. J. Cancer. Res*., **88**, 363–368.
13. Brentnall,T.A., Crispin,D.A., Rabinovitch,P.S., Haggitt,R.C., Rubin,C.E., Stevens,A.C. and Burmer,G.C. (1994) Mutations in the *p53* gene: an early marker of neoplastic progression in ulcerative colitis. *Gastroenterology*, **107**, 369–378.
14. Ilyas,M. and Talbot,I.C. (1995) p53 expression in ulcerative colitis: a longitudinal study. *Gut*, **37**, 802–804.
15. Donehower,L.A., Harvey,M., Slagle,B.L., McArther,M.J., Montgomery, C.A., Butel,J.S. and Bradley,A. (1992) Mice deficient for *p53* are developmentally normal but susceptible to spontaneous tumors. *Nature*, **356**, 215–221.
16. Tarmin,L., Yin,J., Harpaz,N., Kozam,M., Noordzij,J., Antonio,L.B., Jiang,H.Y., Chan,O., Cymes,K. and Meltzer,S.J. (1995) Adenomatous polyposis coli gene mutations in ulcerative colitis-associated dysplasias and cancers versus sporadic colon neoplasms. *Cancer Res*., **55**, 2035–2038.
17. Powell,S.M., Zilz,N., Beazer-Barclay,Y., Bryan,T.M., Hamilton,S.R., Thibodeau,S.N., Vogelstein,B. and Kinzler,K.W. (1992) *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359**, 235–237.
18. Leach,F.S., Nicolaides,N.C., Papadopoulos,N. *et al*. (1993) Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75**, 1215–1225.
19. Kim,H., Jen,J., Vogelstein,B. and Hamilton,S.R. (1994) Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *Am. J. Pathol*., **145**, 148–156.
20. Suzuki,H., Harpaz,N., Tarmin,L., Yin,J., Jiang,H., Bell,J.D., Hontanosas,M., Groisman,G.M., Abraham,J.M. and Meltzer,S.J. (1994) Microsatellite instability in ulcerative colitis-associated colorectal dysplasias and cancers. *Cancer Res*., **54**, 4841–4844.
21. Brentnall,T.A., Crispin,D.A., Bronner,M.P., Cherian,S.P., Hueffed,M., Rabinovitch,P.S., Rubin,C.R., Haggitt,R.C. and Boland,C.R. (1996) Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res*., **56**, 1267–1240.
22. Brentnall,T.A., Rubin,C.E., Crispin,D.A., Stevens,A., Batchelor,R.H. and Haggitt,R. (1995) A germline substitution in the human *MSH2* gene is associated with high-grade dysplasia and cancer in ulcerative colitis. *Gastroenterology*, **109**, 151–155.
23. Loeb,K.R. and Loeb,L.A. (1999) Genetic instability and the mutator phenotype. Studies in ulcerative colitis. *Am. J. Pathol*., **154**, 1621–1626.
24. Tsushima,H., Kawata,S., Tamura,S., Ito,N., Shirai,Y., Kiso,S., Imai,Y., Shimomukai,H., Nomura,Y., Matsuda,Y. and Matsuzawa,Y. (1996) High levels of transforming growth factor β1 in patients with colorectal cancer: association with disease progression. *Gastroenterology*, **110**, 375–382.
25. Engle,S.J., Hoying,J.B., Boivin,G.P., Ormsby,I., Gartside,P.S. and Doetschman,T. (1999) Transforming growth factor β1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res.*, **59**, 3379–3386.
26. Manning,A.M., Williams,A.C., Game,S.M. and Paraskeva,C. (1991) Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor β: conversion of an adenoma cell line to a tumorogenic phenotype as accompanied by reduced response to the inhibitory effects of *TGF*-β. *Oncogene*, **6**, 1471–1476.
27. Eppert,K., Scherer,W.S., Ozcelik,H. *et al*. (1996) MADR maps to 18q21 and encodes a TGF-β-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552.
28. Takagi,Y., Kohmura,H., Futamura,M., Kida,H., Tanemura,H., Shimokawa, K. and Saji,S. (1996) Somatic alteration of the DPC4 gene in human colorectal cancers *in vivo*. *Gastroenterology*, **111**, 1369–1372.
29. Howe,J.R., Roth,S., Ringold,J.C. *et al*. (1998) Mutations in the *SMAD4/ DPC4* gene in juvenile polyposis. *Science*, **280**, 1086–1088.
30. Taketo,M.M. and Takaku,K. (2000) Gastro-intestinal tumorigenesis in *Smad4* mutant mice. *Cytokine Growth Factor Rev.*, **11**, 147–157.
31. Zhu,Y., Richardson,J.A., Parada,L.F. and Graff,J.M. (1998) *Smad3* mutant mice develop metastatic colorectal cancer. *Cell*, **94**, 703–714.

Received August 24, 2000; revised December 29, 2000; accepted January 5, 2001