Fiber-specific Molecular Features of Tumors Induced in Rat Peritoneum

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Molecular markers such as mutational spectra or mRNA expression patterns may give some indication of the mechanisms of carcinogenesis induced by fibers and other carcinogens. In our study, tumors were induced by application of crocidolite asbestos or benz[a]pyrene (BaP) to rat peritoneum. DNA and RNA of these tumors were subjected to analysis of point mutations and to investigation of mRNA expression patterns. With both assays we found typical features depending on the type of carcinogen applied. The analysis of point mutations in the tumor suppressor gene p53 revealed mutations in the BaP-induced tumors. However, in the tumors induced by crocidolite asbestos that were of the same tumor type as those induced by BaP, mutations in p53 were not detectable. Every mutation detected on the DNA level causes an amino acid substitution within one of the functional domains of the tumor suppressor protein. Therefore, these mutations seem to be of biological relevance for tumor progression and indicate a difference in the carcinogenesis regarding the type of the carcinogenic substance. An additional specificity of crocidolite-induced tumors was detectable by analyzing the mRNA expression of the tumor suppressor gene WT1, which is known to be expressed in human mesothelial and mesothelioma cells. A relatively high amount of WT1 mRNA was measured by quantitative competitive reverse transcription-polymerase reaction using RNA extracted from crocidolite-induced tumors. However, WT1 seems to be expressed on a rather low level in tumors induced by BaP.

Key words: asbestos, benz[a]pyrene, intraperitoneal injection, malignant mesothelioma, p53, WT1

Introduction

Tumors can be induced in the peritoneal cavity of rats by application of fibrous dusts. These tumors arise from mesothelial cells and are histologically comparable with the mesotheliomas induced by fibers in human pleura (1). This experimental system of ip injection has been used as a very sensitive assay for the assessment of fiber carcinogenicity (2,3). Since the general mechanism of tumor induction by fibers is still not understood, the relevance of the data obtained in the animal experiments used to assess fiber carcinogenicity in humans is discussed in depth (4). Therefore, it is important to elucidate the mechanisms of fiber carcinogenesis. Molecular characterization of the tumors induced by fibers in rats may help in comparing them with tumors occurring in humans.

The investigation of oncogenes and tumor suppressor genes may provide information about tumor characteristics and the mechanisms of carcinogenesis induced by fibers. Mutational spectra of the p53 gene can give information about the way carcinogenic substances interact with the DNA (5,6). Most of the mutations that inactivate gene function occur within five functional domains of the gene (I–V) that contain the information for the DNA-binding activity of the protein (7). The position and type of mutation (transition or transversion) can leave a "molecular footprint" on the DNA (8). One hypothesis concerning the carcinogenic effect of fibers is based on the involvement of reactive oxygen metabolites (9). Reactive oxygen species (ROS) may be released by macrophages that accumulate in chronic inflammatory processes induced by fibers (10,11). A reaction of ROS with DNA may result in the generation of point mutations and therefore may be the cause of well-defined point mutations in p53, as described for H2O2 (12). p53 is well known as a regulator of the cell cycle (13) as well as a protein that is activated and accumulated when DNA-damaging agents are applied (14). The occurrence of point mutations in p53 and the differences in the expression levels of this tumor suppressor may be a typical feature of a tumor type and may give insight into the development of a malignant tumor.

Point mutations in p53 and the expression of p53 mRNA in cultured human mesothelioma cells have been investigated in two studies (15,16). Collectively the results showed that the occurrence of point mutations in p53 seems of minor importance in human mesotheliomas compared with other human tumor types. These results, however, preclude a statement about the potential of fibers to induce point mutations. In the case of human mesothelioma, p53 may not be the appropriate target for mutations to occur.

The Wilms' tumor suppressor gene (WT1) coding for a DNA-binding zinc finger protein was first identified by its mutational inactivation in cases of Wilms' tumors (17–19). The normal expression pattern in several fetal and adult mesoderm-derived tissues exhibits variations in the amount of mRNA from four possible splicing variants (20). WT1 expression can be observed in mesothelial cells from humans, mice, and rats, as well as in pleural mesothelioma cells from humans and rats (21,22). In one case of human mesothelioma, a mutation in WT1 was observed that converted the protein function from a transcriptional repressor into a transcriptional activator (21). Walker et al. (22) suggest using WT1 expression as a diagnostic marker to distinguish mesothelioma from other tumors occurring in the pleural cavity.

This study compares molecular features such as p53 mutations, as well as mRNA
expression of p53 and WT1 in rat mesothelioma induced by fibers and a chemical carcinogen, to find typical features for fiber-induced carcinogenesis. In addition, similarities to or differences from the human system may allow us to evaluate the experimental system of fiber administration by ip injection in rats. To this end, tumors induced by ip injection of crocidolite asbestos and benzo[a]pyrene (B[a]P) were subjected to analysis of mutations in p53 by denaturing gradient gel electrophoresis (DGGE) and DNA sequencing, and to analysis of p53 and WT1 mRNA expression by competitive reverse transcription-polymerase chain reaction (RT-PCR).

Material and Methods

Animal Experiment

Three groups of rats (Wistar Wi/Wu, Kissleg, Federal Republic of Germany) were treated by ip injection as described for the experiments reported by Roller et al. (4). Crocidolite asbestos (South Africa) (23) was administered in a 2-mg dose per animal. Each dose was suspended in 2 ml of 0.9% NaCl solution. Five milligrams of B[a]P was injected with a carrier substance (1.5 ml beeswax/tricapryline 20:80) not known to induce tumor rates any higher than those in nontreated animals. Control animals were treated with 2 ml of 0.9% NaCl solution. Animals were sacrificed after they exhibited visible health impairment. Their peritoneums were opened and tissue samples of macroscopically visible tumors, as well as of several other tissues, were taken. Each sample was divided into two parts. One part was frozen in liquid nitrogen and kept at −70°C for later preparation of genomic DNA and total RNA. The other part was fixed in formalin and embedded in paraffin for histologic investigations as described by Friemann et al. (24).

DNA Preparation

DNA extraction of tumor and nontumor tissue was performed according to a commercially available DNA extraction kit (Stratagene, La Jolla, CA).

DNA Amplification by Polymerase Chain Reaction. Genomic DNA (50 ng) was amplified in a first polymerase chain reaction (PCR) using the following primer combinations: p53IIRL/p53IIbRR (half of domain II and domain III); p53IRL/p53IbRR (domain IV); and p53IIbRL/p53IIbRR (domain V) (25). Amplified DNA fragments contained intron sequences of the primers were selected to anneal within introns to ensure that sequences of pseudogenes that may exist in the rat genome were not amplified or could be discriminated. Primer sequences were p53IIbRL 5'-CCGCATCTTTGATGATTCT-3'; p53IIbRR 5'-TTGAAACCAGTTCTACCCCA-3'; p53IIbRL 5'-TCCGACTATACCTATCCAC-3'; p53IRL 5'-CCAAGATGCCAGACATAAAGA-3'; p53IIbRL 5'-GGATGGAGCTTCTTCT-3'; p53RR 5'-AGCGGCGACATGTCTTTT-3'. In each PCR one of the primers contained an eight-base attachment site at the 5' end for subsequent incorporation of a GC clamp (26).

Standard PCR conditions using a hot start technique were 30×45 sec at 94°C, 45 sec at 50°C, 1 min at 72°C for the PCR with p53IIRL/p53IIbRR and p53IRL/p53IbRR, for p53IIbRL/p53IIbRR PCR conditions were similar except for the annealing temperature (1 min at 55°C). Attachment of the GC clamp was accomplished by reamplifying aliquots of PCR products according to Sheffield et al. (26). To generate heteroduplex molecules after PCR, we performed a denaturing step of 10 min at 95°C and an annealing by continuously cooling to room temperature over 30 min. PCR products were precipitated and subsequently resolved in 10 mM Tris-Cl, pH 8, 1 mM EDTA; approximately 100 ng were analyzed by DGGE.

Denaturing Gradient Gel Electrophoresis. Five percent polyacrylamide gels with a gradient ranging from 20 to 80% denaturing agents parallel to the direction of electrophoresis [100% denaturant: 7 M urea/40% formamide; (27)] were run at 60°C with 2000 Vh (p53IIRL/p53IIbRR and p53IIbRL/p53IIbRR), or 3200 Vh (p53IRL/p53IbRR). DNA was made visible by a silver staining method (28).

Sequence Analysis. Templates for the sequencing reactions were generated in a separate PCR with the above-mentioned primer combinations lacking the attachment region. Direct sequencing of the PCR products was performed by modification of the dye deoxy chain termination method of Sanger et al. (29) using the PCR primers.

RNA Preparation and Competitive Polymerase Chain Reaction

Preparation of total RNA was performed according to Chomczynski et al. (30). Rat p53 cDNA fragment (219 bp) containing domains IV and V were amplified using p53RL and p53RR. A competitive reference standard (CRS) with a deletion of 50 bp was constructed as described (31). Using the same technique, a rat β-actin CRS (246 bp) lacking 41 bp and a WT1 CRS amplified from the shorter splice variant of alternative splice I (131 bp) lacking 13 bp were not generated. Primers for β-actin were Acct3'GGGCGGACTGTCATACCTC-3' and Acct5'GTTGGCATCAGAAACTAC-3'. Primers for WT1 alternative splice I are 106 and 26 according to Haber et al. (20) with the species-specific sequence changes. In additional PCR the three fragments were subsequently linked to obtain internal standards that can be used in identical concentrations in separate PCR for WT1 and β-actin (32). RT–PCR was performed as described (33) with 0.25 fmol CRS and 5×10−2 to 5×10−5 dilutions (8 steps) of the cDNA. PCR products were run on 10% polyacrylamide gels with 1×0.9 M Tris–borate, 0.002 M EDTA, pH 8. Quantification was performed after staining with ethidium bromide and photograph documentation. After validation of the method by scanning gel images using a OmniMedia Scanner XRS and a Biomager System (Millipore, Ann Arbor, MI), band intensities were compared visually. In the case of WT1, the intensity of the 131-bp band was compared to the internal standard molecule. Semiquantitative RT–PCRs were performed under almost similar conditions. No CRS was added in these reactions and the amplification was performed with 25 to 28 cycles (data not shown). Primers for HPRT were HPRTS'5'-GCTGGTGAAAGACCTCT-3' and HPRT3'5'-ACAGGACTAGAACTGTC-3'.

Results

An animal experiment was performed to provide tumors for mutational analysis and mRNA expression analysis. Tumors were induced by ip injection of crocidolite asbestos or B[a]P in separate groups. The control group was treated with physiologic NaCl solution. Table 1 summarizes the tumor data obtained from the three experimental groups. The distinction between induced and spontaneous tumors was made according to Roller et al. (4). The results of the histopathologic investigations on these tumors are published elsewhere (24). There were no differences in the morphologic differentiation of the tumors among the samples from animals treated with different carcinogens.

As a prescreening assay, we chose DGGE which is well known as a reliable method for rapid detection of point mutations (34). The heteroduplex-DGGE we applied uses a GC clamp on one side of the
PCR product and detects mismatched base pairs (26). The GC clamp ensures the partial denaturation and the formation of a Y-shaped structure of the DNA double-strand during electrophoresis on a gel containing a denaturing gradient. The migration velocity of these Y-shaped molecules decreases and a banding at the corresponding concentration of denaturing agents takes place. Because of wild-type sequences that are present in our PCR and denaturing and reannealing steps after PCR, the amplification of mutated sequences results in formation of a DNA double-strand containing at least one base pair that is mismatched. The mismatched base pair causes a melting behavior that differs significantly from that of the DNA without a mismatched base pair (27). Examples for the three different PCR products used for the examination of the p53 functional domains are shown in Figure 1. In the analysis of domains II/III and IV, both homoduplexes and the two homoduplexes are visible as separate bands. Only one band for both homoduplexes is visible in the case of the point mutation in domain IV in DNA from animal T212/93. Here the melting temperature of the homoduplexes does not differ. For most tumors, several DNA samples from different parts of the tumor were prepared and examined. In addition, one sample of nontumorous tissue (mostly liver) from each animal was analyzed. Interestingly, we found eight point mutations in the tumors induced by B[a]P, whereas no mutations were detectable in the crocidolite group (Table 1). A point mutation occurred in one carcinoma of the ovary from the control group (a tumor type that was not examined in the B[a]P and crocidolite groups).

Sequence analysis was performed to determine the type and the exact position of the point mutations and to verify the method of DGGE. DGGE was verified by sequencing 37 DNA fragments that exhibited no additional bands in the DGGE (data not shown). In these DNA samples no mutations were detectable by sequence analysis and, conversely, every mutation occurring in the DGGE resulted in an altered DNA sequence in the sequencing analysis. Sequencing reactions were performed with newly amplified DNA fragments to ensure that errors of the Taq polymerase did not cause false positives. Only mutations occurring in both assays were assessed to be positive. Table 2 summarizes the locations and the types of the mutations found with both assays. Obviously, all mutations observed result in the substitution of an amino acid or, as in one case, in the formation of a stop codon.

To analyze the expression of p53 mRNA and WT1 mRNA competitive RT–PCR using an internal mimic molecule, CRS, was performed. As a positive control for RT–PCR and to test the quality of the mRNA, the amount of β-actin mRNA was determined for each RNA sample. An additional positive control was made by measuring HPR7 mRNA expression. Semiquantitative RT–PCRs for β-actin and HPR7 were compared. Expression levels of both housekeeping genes correlated well (data not shown). Examples of quantifications of β-actin and WT1 cDNA of a B[a]P- and a crocidolite-induced tumor are depicted in Figure 2. The results obtained from mRNAs of 14 tumors induced by B[a]P and of 9 tumors induced by crocidolite asbestos are summarized in Table 3. Obviously, p53 mRNA can be detected in all of the samples investigated regardless of the tumor-inducing agent. However, the amount of mRNA varies from approximately 1 to 100 fmol/μg RNA. In the tumors with mutations in the p53 gene, mRNA transcribed from this gene is detectable in considerable amounts. An additional sequence analysis of the amplified cDNA of these tumors showed that the mRNA sequence refers to the mutated type (data not shown). Results from WT1 expression analysis revealed low amounts of WT1 cDNA, ranging from 1 to 5 fmol/μg RNA in the B[a]P group. However, in the crocidolite group, except...
Figure 2. Competitive RT–PCR of β–Actin and WT1 mRNA. Dilutions of cDNA were amplified under competitive conditions in the presence of a mimic molecule (CRS). The lower bands refer to the CRS. In WT1 PCR's two bands referring to the alternative splice I are visible. Band intensities reflect the molar ratio of cDNA and CRS at the onset of the PCR. T200/93, tumor from a B[a]P-treated animal. T310/93, tumor from a crocidolite-treated animal.

Table 3. β–Actin, p53, and WT1 mRNA amounts measured by competitive RT–PCR.

| Carcinogen      | Animal number | β–Actin cDNA, fmol/µg | p53 cDNA, fmol/µg | WT1 cDNA, fmol/µg |
|-----------------|---------------|------------------------|-------------------|-------------------|
| Benzo[a]pyrene  | T199/93       | 35                     | 35                | 1                 |
| Benzo[a]pyrene  | T200/93       | 5                      | 7.5               | 2.5               |
| Benzo[a]pyrene  | T201/93a      | 35                     | 35                | 5                 |
| Benzo[a]pyrene  | T202/93       | 2.5                    | 2.5               | 1                 |
| Benzo[a]pyrene  | T203/93a      | 75                     | 100               | 1                 |
| Benzo[a]pyrene  | T204/93a      | 10                     | 7.5               | 2.5               |
| Benzo[a]pyrene  | T208/93a      | 25                     | 75                | 5                 |
| Benzo[a]pyrene  | T209/93       | 35                     | 1                 | 2.5               |
| Benzo[a]pyrene  | T212/93a      | 35                     | 50                | 5                 |
| Benzo[a]pyrene  | T217/93a      | 50                     | 50                | ND                |
| Benzo[a]pyrene  | T218/93       | 75                     | 50                | ND                |
| Benzo[a]pyrene  | T219/93       | 10                     | 35                | 1                 |
| Benzo[a]pyrene  | T219/93b      | 10                     | 35                | 1.75              |
| Benzo[a]pyrene  | T222/93a      | 10                     | 35                | 1                 |
| Benzo[a]pyrene  | T226/93a      | 50                     | 35                | 1                 |
| Crocidolite asbestos | T295/93     | 75                     | 10                | 75*               |
| Crocidolite asbestos | T300/93      | 5                      | 35                | 35*               |
| Crocidolite asbestos | T303/93      | 5                      | 5                 | 35*               |
| Crocidolite asbestos | T305/93      | 2.5                    | 5                 | 10*               |
| Crocidolite asbestos | T309/93      | 35                     | 0.5               | 0.5               |
| Crocidolite asbestos | T310/93      | 2.5                    | 4                 | 10*               |
| Crocidolite asbestos | T317/93      | 50                     | 50                | 50*               |
| Crocidolite asbestos | T318/93      | 2.5                    | 35                | 10*               |
| Crocidolite asbestos | T321/93      | 50                     | 5                 | 75*               |

ND, not determined. *RNA from tumorous tissue with detected mutations in p53. **High expression levels of WT1 mRNA in crocidolite-induced tumors.

in one tumor, relatively high amounts above 10 fmol/µg RNA can be observed.

**Discussion**

Analysis of mutations in the tumor suppressor gene p53 in tumors induced by ip injection of B[a]P and crocidolite asbestos revealed significant differences depending on the kind of carcinogen applied. All mutations detected in the B[a]P-induced tumors resulted in substitutions of an amino acid or in the formation of a stop codon that leads to truncated mRNA molecules (Table 2). The sequencing analysis of the RT–PCR products showed that the mutant mRNAs are detectable in these tumors. These results suggest that in these tumors the mutant p53 tumor suppressor molecule leads to an escape from cell cycle control mediated by the wild-type p53 molecule (35). Mutation of p53 seems to be a common event in the tumorigenesis of this tumor type when it is induced by B[a]P. Interestingly, in tumors induced by crocidolite asbestos, no p53 point mutations were detectable (Table 1). Since a large number of tumor samples was examined and the reliability of the DGGE prescreening method could be demonstrated, we can consider this result representative of tumors induced by ip injection in rats. Spontaneously occurring mutations, mostly C–T transitions that arise from spontaneous deamination of naturally occurring 5-methylcytosin residues (36), also were not detectable in our fiber-induced tumors. This result may be of interest with regard to the problem of carcinogenesis induced by mitogenic substances. Several publications discussed controversially a mechanism of carcinogenesis induced by proliferation-inducing agents (37–39). Asbestos (or fibers in general) is sometimes classified as a mitogenically acting carcinogen (37). The rationale for this is that because of the high replication rate induced by this kind of substance, naturally occurring mutations accumulate in the exposed tissue. The probability of spontaneous mutations should rise with the increase of cell divisions, as observed in tumor proliferation. In this case, we would have expected at least a low frequency of spontaneous p53 mutations in the crocidolite group. If we hypothesize a high amount of cell proliferation in all tumors regardless of the kind of inducing agent, our results show that—at least for p53 in this experimental system—there is no indication of such a mechanism.

The complete lack of p53 mutations in the crocidolite-induced tumors may also be explained by differences in the mechanisms involved in tumor development. Kinzler and Vogelstein (40) suggested, interpreting p53 expression and mutation data from tumor cells under hypoxia according to Graeber et al. (41), that under certain conditions in a developing tumor, p53 may be induced and cell cycle control may then be mediated by the p53 tumor suppressor protein. In this situation, cells-bearing
mutations in p53 escape from the cell-cycle control and achieve a growth advantage. We hypothesize that in our experimental system such a mechanism that induces p53 expression, may take place in the tumors induced by B[a]P. However, in the tumors induced by crocidolite asbestos, the escape from cell-cycle control may be mediated by another mechanism not involving p53. In this case, the mutations observed would be the result of a selection mechanism due to induction of p53 in a certain stage of tumor development. On the other hand, in the crocidolite group no point mutations attributable to this selection mechanism could be detected.

In human mesotheliomas, p53 mutations are of minor importance in this tumor type (15,16). Mutations were detectable in 4 cases of 24 cell lines. We believe the situation is similar in both systems of fiber carcinogenesis. The lack of mutations in the animal system may be explained by the well-defined experimental system. In human samples, additional carcinogens may have induced mutations in p53. It is obvious that the mutational status of p53 could be useful to distinguish whether fibrous or nonfibrous agents caused a tumor. However, for this kind of diagnosis a large amount of data, especially from human mesothelioma of patients with well-known exposures, would be necessary.

Further carcinoagenic-specific features could be detected by the analysis of the W71 mRNA amount using competitive RT–PCR. As in human mesotheliomas and rat pleural mesotheliomas, we found mRNA transcribed from this gene in all tumors investigated (Table 3). Differences in the amounts of splice variants could not be observed (data not shown). However, the extent of mRNA differed between the two experimental groups. In B[a]P-induced tumors a relatively lower amount of W71 mRNA was detected than in the crocidolite-induced tumors. The biological relevance of these differences may be explained by some characteristics of the W71 zinc finger protein. The mRNA transcription of some growth factors known to be overexpressed in mesothelioma is normally repressed by binding of W71 to the gene consensus sequence EGR1 (42,43). In mesothelioma cells with W71 inactivated by mutations, the expression of these growth factors may be a growth stimulus for the tumor cell (44). Because only a few mesotheliomas with W71 are described in the literature, this pathway of tumorigenesis apparently does not occur frequently. Another feature of the W71 protein is its affinity to p53. Both gene products can form a heterodimer protein complex that exhibits altered protein functions (45). W71 then acts as a transcriptional activator after binding to the consensus sequence of the target gene. In the presence of certain amounts of W71 protein, a stabilization of p53 protein and an inhibition of apoptosis was observed (46). Further investigation of the ip-induced tumors will determine whether one of these mechanisms is in force in fiber-induced tumors.

The results presented here show parallels between human mesotheliomas and the tumors induced by ip application of crocidolite asbestos in rats. In both systems p53 mutations seem to be of minor importance. Because of the positive results revealed from the B[a]P-induced tumors, the lack of mutations in p53 does not appear to be a characteristic of the tumor type mesothelioma. Moreover, a normal p53 status may be a typical feature of crocidolite-induced carcinogenesis and possibly of fiber-induced carcinogenesis in general.

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