Carcinogenesis vol.21 no.5 pp.1023–1029, 2000

mRNA expression patterns in different stages of asbestos-induced carcinogenesis in rats

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Human malignant mesotheliomas are induced almost exclusively by fibrous dusts. The nature of interactions between fibers and target cells, and the molecular mechanisms leading to tumorigenesis, are not yet understood. Here, the mRNA expression patterns at different stages of asbestos-induced carcinogenesis in rats were monitored by suppression subtractive hybridization (SSH) and array assay. Several genes were upregulated in pretumorous tissues from asbestos-treated rats, in asbestos-induced tumors and in cells treated with asbestos *in vitro*. The upregulation of the proto-oncogene c-*myc*, *fra-1* and *egfr* in fiber-induced carcinogenesis was demonstrated at different stages of carcinogenesis. A possible role of Fra-1 as one of the dimeric proteins generating the AP-1 transcription factor was substantiated by its dose-dependent expression in mesothelial cells treated with asbestos *in vitro*. The upregulation of osteopontin (an extracellular matrix protein) and of zyxin and integrin-linked kinase (intracellular proteins associated with the focal adhesion contact), indicate that fibers may affect integrin-linked signal transduction and extracellular matrix proteins.

Introduction

The mechanism of carcinogenicity of fibrous dusts and isomorphic particles is not yet understood. These carcinogenic substances do not act in the same way as chemicals with well-known mutagenic or clastogenic activity. It has been suggested that fibers interact physically with structures in target cells (1). Genetic damage may be involved in these processes: fiber-induced oxygen free radicals or direct fiber–chromosome interactions may result in the activation of oncogenes or in the inactivation of tumor suppressor genes. There is also some evidence for indirect effects mediated by inflammatory cells (1). Phagocytosis of the fibers can elicit the release of important modulators of cell growth and differentiation.

Malignant mesothelioma in humans is almost exclusively caused by asbestos or fibrous dusts with particular physical properties (2). Inhaled fibers penetrate the lung tissue and accumulate in the pleural space (3). In the mesothelial cell layer lining the pleural cavity, fibrosis and hyperplasia are induced. After a long period of latency, tumors can occur in this area.

The mechanisms of mesothelioma tumorigenesis are currently investigated using different *in vitro* experimental systems. Tumor cell lines of humans and rodents have been analyzed for the expression of growth factors and the corresponding receptors that may contribute to tumor formation and to tumor progression. Comparison of cell lines from tumors induced by asbestos with spontaneously immortalized cell lines can provide useful information that can be correlated with fiber-specific tumorigenesis (4). Autocrine growth loops consisting of transforming growth factor alpha (TGF-α) and the epidermal growth factor receptor (EGFR), as well as insulin-like growth factor II (IGF II) and the corresponding receptor, have been shown to be relevant only in cell lines from asbestos-induced tumors but not in spontaneously transformed cell lines (4,5).

Early cellular responses to fibers that may lead to the induction of tumorigenesis are investigated using rat pleural mesothelial (RPM) cells treated with several kinds of fibrous and non-fibrous dusts. Cell proliferation and apoptosis are induced simultaneously by the application of fibers (6).

Several mechanisms of signal transduction, including mitogen-activated protein kinases (MAPKs) resulting in activation of the AP-1 transcription factor, are involved in these cellular reactions (7). However, the initiating steps of fiber–cell interaction leading to the induction of signal transduction cascades are still not understood. Signal transduction pathways in response to fiber stress, as well as the autocrine regulation observed in mesothelial cell lines, have not been demonstrated *in vivo*. Such information, however, is important to identify general principles of fiber-induced carcinogenesis.

The aim of this study was to identify cellular reactions *in vivo* associated with crocidolite fiber-induced carcinogenesis. Analysis of signal transduction pathways affected by fiber stress may help us to understand the processes that lead to dysregulation of the cell cycle and, hence, carcinogenesis. Moreover, fiber–cell interactions that lead to the induction of these processes should be monitored with this approach.

A second aim of this study was to evaluate the *in vivo* system of intraperitoneal (i.p.) injection in rats (8,9) to investigate fiber-induced carcinogenesis. Using this experimental model system, we demonstrated specific mRNA patterns in different stages of mesothelioma carcinogenesis.

Materials and methods

Animal treatment and tissue samples

Tumor tissue for suppression subtractive hybridization (SSH) came from an animal experiment described already (10). Briefly, 8-week-old Wistar rats were treated by i.p. injection with a single dose of 2 mg crocidolite asbestos from South Africa with a 59% fiber fraction of >5 μm (length:diameter ratio >5:1; fiber size distribution described in ref. 11) suspended in 2 ml 0.9% NaCl solution per animal. Control animals were subjected to the i.p. application of 2 ml carrier solution. An additional control group was given, as described

Abbreviations: EGFR, epidermal growth factor receptor; Fra-1, Fos-related antigen-1; IGF II, insulin-like growth factor II; RPM, rat pleural mesothelial; SSH, suppression subtractive hybridization; TGF-α, transforming growth factor alpha.

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in ref. 10, a single i.p. injection of 25 mg granular silicon carbide (SiC) suspended in 2 ml 0.9% NaCl. The sample had the following chemical and physical properties: SiC, 97.6%; C (free), 0.28%; Si (free), 0.34%; SiO_2 (free), 1.02%; Fe (total), 0.048%; 95% of particles were <3 µm, 70% <2 µm, 40% <1 µm and 20% <0.6 µm. Animals were killed when their health deteriorated visibly. Tumors developed 70–110 weeks after treatment. Control animals were killed after 100 weeks. Macroscopically visible tumors in the greater omentum were prepared, snap frozen and stored at –80°C for later preparation of nucleic acids. Omenta from control animals were treated in the same way. Part of each sample was investigated histologically. The tissue samples used for SSH were selected under pathological control. No tumor induction was seen in SiC-treated animals. For SSH, animals treated with saline alone were used as controls.

To provide pretumorous tissue samples for the array assay, another animal experiment was performed. Conditions (animal strain, substances and dosages) were as described above, but animals were killed 52 weeks after treatment. Pathological examination did not reveal any tumors at this time point. Control animals were treated with carrier solution and killed at the same time.

RNA preparation

Total RNA was prepared using TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's advice, homogenizing tissues directly with the reagent. Poly(A)$^+$ mRNA was isolated using oligo(dT)-coated latex particles (Oligotex mRNA kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Suppression subtractive hybridization

Suppression subtractive hybridization (12) was performed using a PCR-Select cDNA subtraction kit (Clontech Laboratories, Palo Alto, CA). Two micrograms of poly(A)$^+$ mRNA, pooled from equal amounts of mRNA from tumors from six animals, was used to produce the tester cDNA. Two micrograms of poly(A)$^+$ mRNA, pooled from equal amounts of mRNA from omenta of seven control animals, was used to produce the driver cDNA. After ligation of the adapters and a denaturation step, the tester cDNA fragments were allowed to hybridize with an excess of driver cDNA not bearing adapters. cDNA was then amplified by PCR using adapter sequences as primer targets.

PCR products, thus enriched in mRNA sequences upregulated in tumors, were cloned directly by blunt-end ligation into pCR-TRAP vector (GenHunter, Nashville, TN) according to the manufacturer's instructions. Colonies growing on selection medium were checked for DNA inserts by colony PCR, as recommended.

DNA sequencing

Plasmid DNA from positive clones was prepared from 100 ml overnight cultures using the QIAfilter Plasmid Midi Kit (Qiagen). DNA sequences of plasmid inserts were determined using an ALFexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden). Sequencing reactions were performed with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) using fluorescently labeled primers (Lseq and Rseq from the cloning kit).

Sequences were analyzed for similarities to known sequences in the DNA databases available at the National Center for Biotechnology Information (Bethesda, MD). The BLAST search option was used via the World Wide Web.

Array assay

The Atlas cDNA expression array (Clontech) was used according to the manufacturer's protocol. Two identical nylon membranes loaded with 588 different human cDNA samples were subjected to parallel hybridization with test cDNA and control cDNA. To prepare hybridization probes representing the mRNA status of pretumorous asbestos-treated omenta and of control omenta, equal amounts of mRNA from seven treated animals and five control animals, respectively, were pooled before cDNA synthesis. Poly(A)$^+$ mRNA (0.2 µg) was subjected to cDNA synthesis in the presence of [α-^{32}P]dATP using the array kit. Hybridization was performed as recommended in the kit protocol. Hybridization signals were visualized by placing the membranes against X-ray film. The signals obtained from the two independent hybridizations were compared to select cDNAs that differed in expression.

Dot blot hybridization

Plasmid DNA (5 µg) was spotted on to two separate sheets of nylon membrane (Hybond-N+; Amersham Pharmacia Biotech) according to the manufacturer's alkaline blotting procedure. Aliquots of cDNA (100 ng) were labeled with the Ready To Go labeling kit (Amersham Pharmacia Biotech) using [α-^{32}P]dCTP. Probes from driver and tester cDNA with similar specific radioactivity were used for separate stringent hybridization of the membranes, according to the hybridization protocol provided with the membrane.

Semiquantitative RT–PCR

For reverse transcription, 500 ng of total RNA were subjected to reverse transcription primed by oligo(dT) with AMV reverse transcriptase (Promega, Madison, WI) according to the recommendations of the manufacturer in a total volume of 50 µl. One microliter of a 1 in 10 dilution of cDNA was subjected to PCR using *Taq* DNA polymerase (Roche, Mannheim, Germany). To ensure analysis of PCR products in the logarithmic phase of amplification, several parallel reactions with increasing cycle numbers were performed. As a control for cDNA synthesis, cDNA of constitutively expressed genes *gapdh* and β-*act* (encoding glyceraldehyde-3-phosphate-dehydrogenase and β-actin, respectively) was amplified. PCR conditions were as follows: a hot start after 5 min at 94°C; *n* cycles consisting of (i) denaturing for 30 s at 94°C; (ii) annealing for 30 s (see below for temperature); (iii) elongation for 75 s at 72°C. The number of cycles (*n*), annealing temperature, fragment length and primer sequences are listed in Table I. Significant differences in band intensity in ethidium bromide-stained agarose gels were assessed as differences in mRNA expression.

Cells and cell culture

The non-tumorous rat pleural mesothelial cell line 44R.M.-4 (13) was purchased from the European Collection of Cell Cultures (Salisbury, UK), and cultured in Ham's F12 medium supplemented with 15% fetal bovine serum.

Primary RPM cells were prepared from Wistar rats, between 8 and 10 weeks of age, as described by Jaurand *et al.* (14). The cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, hydrocortisone (100 ng/ml), insulin (2.5 µg/ml) and selenium (2.5 ng/ml). For *in vitro* experiments, cells were used after passaging one to three times. Only cells from one preparation were used for parallel experiments.

The rat mesothelioma cell line RZ 328 was established from a tumor induced by crocidolite treatment in the animal experiment described above (10) and was kindly donated by Prof. Dr Füzesi (Institute of Pathology, University of Aachen, Germany). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum. All cell culture media, sera and supplements were purchased from Sigma (St Louis, MO).

Cell treatment

Cells were grown until confluence was reached. They were shifted to low serum conditions (1% fetal bovine serum) 16 h before treatment. Asbestos fibers were suspended in phosphate-buffered saline by sonication. The fiber suspension was added to cells at the concentrations indicated.

Results

In order to identify changes in mRNA expression induced by crocidolite asbestos during mesothelioma development, two assays were performed on tumorous and pretumorous tissues.

Genes upregulated in mesothelioma

In the first set of experiments, the endpoint of tumor development was analyzed. Suppression subtractive hybridization (SSH) was used to compare tumors induced by i.p. administration of crocidolite asbestos with control tissue. cDNA from control tissues was used as a driver, and cDNA from tumors as tester. With this experimental design, mRNA sequences upregulated in tumors were enriched. In total, 93 plasmid clones were obtained. To eliminate false positives and cloned non-coding sequences, comparative dot blot hybridization and DNA sequence analysis were performed (data not shown). Five cDNA clones showed clear differences in hybridization signals and were of considerable insert length. A search of databases revealed identities to the sequences of genes for rat osteopontin (*opn*), mouse zyxin (*zyx*) and rat branched chain aminotransferase (*bcat*). Two clones (numbers 35 and 49) had ORFs with no homology to sequences in the available databases, but of considerable length. These data are summarized in Table IIA.

Verification in tissues and cell lines

To confirm further the tumor-specific expression of the identified genes, RT–PCR was performed comparing tumor tissues with control tissues, as well as mesothelial and mesothelioma-derived cell lines. Primer target sequences of *opn*, *zyx* and *bcat* for RT–PCR were selected from sequence strings flanking the cloned fragments, to ensure that the genes identified by database searching were monitored. As Figure 1 shows, no

Table I. Primer sequences and PCR conditions

Primer	Number of cycles	Annealing temperature (°C)	Length (bp)	Sequence
gapdh	30	55	983	upper: 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ lower: 5′-CATGTAGGCCATGAGGTCCACCAC-3′
β-*act*	25	55	288	upper: 5′-TGTGGCATCCACGAAACTAC-3′ lower: 5′-GGGCCGGACTCGTCATACTC-3′
Clone 35	26	68	161	upper: 5′-CAACCGTGCACGCCCAGATGCCG-3′ lower: 5′-GAGAAGGTGCAACATGAAGGGCTACG-3′
Clone 49	29	68	372	upper: 5′-GCAGGCTCGTGGGCCAGTGGTAAC-3′ lower: 5′-GCCCCTCTCTGTCTAGCAGGTAGG-3′
zyx	33	62	825	upper: 5′-GTTCAGCCCTGGTGCTCCAAGTGG-3′ lower: 5′-CAGCCGTTGTCATCTGCCTCAATG-3′
opn	30	50	894	upper: 5′-ACGACGATGACGACGGAGACC-3′ lower: 5′-AGAAAGAACAGAAGCGAAATG-3′
bcat	31	58	267	upper: 5′-CCTACTCAACCTCTGCCAGTCTGT-3′ lower: 5′-AGCCGTTCTCTGCCGCCTCACACT-3′
c-*myc*	33	62	722	upper: 5′-TCCCAAAGCCCCAGCCAAGGTTGTGAG-3′ lower: 5′-CACCGCCCACCACCAGCAGCGACTCTG-3′
fra-1	35	63	277	upper: 5′-TACAGTCCCCCTCAGCCCCGACCAG-3′ lower: 5′-GCACCAGCTCAAGGCGTTCCTTCTG-3′
ilk	35	60	292	upper: 5′-GAGCTTCTCCGAGAGCGGGCAGAGA-3′ lower: 5′-TGAGCCGGGGACACTCTTCATTGAA-3′
egfr	35	60	512	upper: 5′-AGCCGTCCTGTCCAACTAT-3′ lower: 5′-TGGCACCAAAGCTGTACTT-3′

Table II. Summary of genes upregulated in asbestos-induced tumors and pretumorous asbestos-treated omenta

(**A**) Genes/sequences upregulated in tumors induced by asbestos (SSH assay)

Positive sequence	Matched sequence	Accession number	Homologies			ORF length (bp)
			Length (bp)	Identity	Matches[a]	
opn	*R. norvegicus* osteopontin mRNA	M99252	171	97%	1155–1326	
zyx	*M. musculus* zyxin mRNA	Y07711	100	95%	2043–2143	
bcat	*R. norvegicus* branched chain aminotransferase mRNA	U35774	200	100%	1169–1369	
Clone 35	–	AF157832[b]	–	–	–	238
Clone 49	–	AF158379[b]	–	–	–	300

(**B**) Genes upregulated in pretumorous tissue treated with asbestos (array assay)

Positive gene	Protein	Accession number
c-*myc*	Myc	V00568
ilk	integrin-linked kinase	U40282
egfr	epidermal growth factor receptor	X00588
c-*jun*	c-Jun	J04111
fra-1	Fos-related antigen-1	X16707

[a]Gene sequence position.
[b]Sequences submitted to GenBank at the National Center of Biological Information (Bethesda, MD).

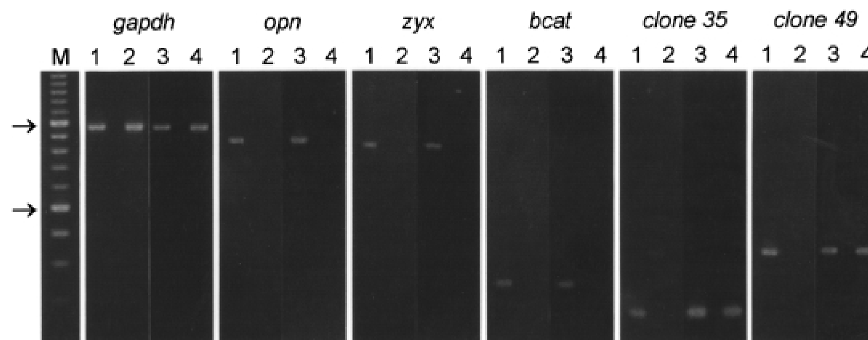

Fig. 1. RT–PCR of the sequences identified by SSH. Samples for cDNA generation were: lanes 1, tumors induced by asbestos; lanes 2, control tissues; lanes 3, the mesothelioma cell line RZ 328; lanes 4, the mesothelial cell line 44R.M.-4. M, molecular weight marker. The arrows indicate bands of 500 and 1000 bp. The *gapdh* gene served as a positive control.

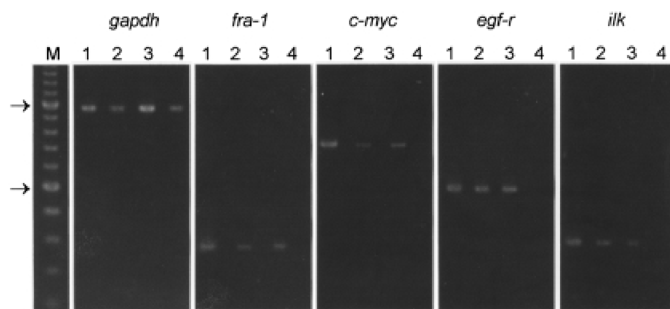

Fig. 2. Expression of genes identified by array assay in tumor samples. Lanes 1, tumors induced by crocidolite asbestos; lanes 2, tumors induced by nickel powder; lanes 3, tumors induced by benzo[*a*]pyrene; lanes 4, control tissues. M, molecular weight marker. The arrows indicate bands of 500 and 1000 bp. The *gapdh* gene served as a positive control.

expression of the tested genes was detected in control tissues, whereas in tumors, RT–PCR revealed enhanced expression of *opn*, *zyx*, *bcat*, clone 35 and clone 49. All five genes were expressed in the mesothelioma tumor cell line RZ 328, whereas in the non-tumorous mesothelial cell line 44R.M.-4, *opn*, *zyx* and *bcat* were not expressed. However, the two ORFs were expressed in this cell line. These sequences may be involved in immortalization, which is necessary for cell-line establishment. RT–PCR analysis of the five genes identified with SSH in 44R.M.-4 cells treated with 10 $\mu g/cm^2$ crocidolite asbestos for 48 h revealed no induction of *opn*, *zyx*, *bcat*, clone 35 or clone 49 mRNA (data not shown). Similar results were obtained when pretumorous tissue from animals treated with asbestos was analyzed (data not shown). These results indicate a specific upregulation of the genes identified by SSH in mesothelioma tumors but not in non-tumorous cells or tissue treated with asbestos.

Genes upregulated in pretumorous tissues

In the second set of experiments, an earlier step of fiber-induced carcinogenesis was investigated. Expression patterns of pretumorous tissues harvested 52 weeks after treatment with asbestos, when no tumors had developed, were compared with control tissues using an array assay based on cDNA hybridization to 588 DNA samples spotted onto membranes. This kind of assay was chosen because it requires relatively little poly(A)$^+$ mRNA as a probe, compared with the SSH assay. Differences in strength of the hybridization signals indicated different mRNA expression rates. False positive results were discriminated by RT–PCR with RNA from pretumorous tissues and control tissues (data not shown).

The results are listed in Table IIB. Genes clearly induced at the mRNA level in the omentum of fiber-treated animals compared with non-treated tissues were c-*myc*, and the *ilk* and *egfr* genes, encoding integrin-linked kinase and epidermal growth factor receptor, respectively. The genes encoding monomeric proteins of the transcription factor AP-1, c-Jun and Fos-related antigen-1 (*fra-1*), revealed enhanced signal strength. The difference in signal strength was greater for *fra-1* than for c-*jun* (data not shown), so only the former gene was investigated further. No genes with a noticeable reduction of mRNA expression in pretumorous tissues were found.

Expression of fra-1, c-myc, ilk and egfr in tumors

To investigate whether the expression of these genes can also be detected in late stages of tumor development, RT–PCR was performed with mRNA from tumors. As shown in Figure 2,

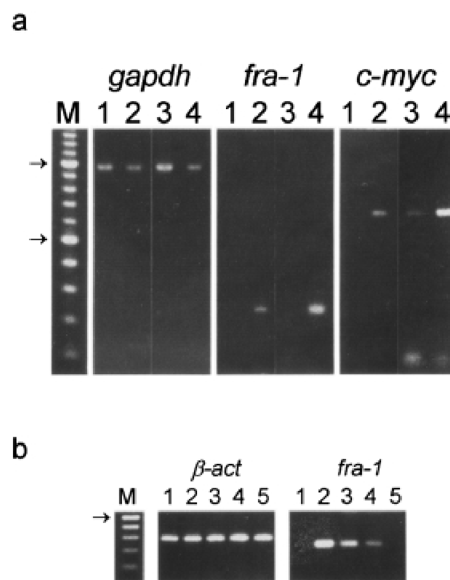

Fig. 3. Expression of *fra-1* and c-*myc* in mesothelial cells treated for 48 h with crocidolite asbestos *in vitro*. (**a**) RT–PCR with mRNA from the mesothelial cell line 44R.M.-4 (lanes 1 and 2) and primary RPM cells (lanes 3 and 4). Lanes 1 and 3, cells treated with carrier substance (PBS); lanes 2 and 4, cells treated with 10 $\mu g/cm^2$ crocidolite asbestos. M, molecular weight marker. The arrows indicate bands of 500 and 1000 bp. The *gapdh* gene served as a positive control. (**b**) RT–PCR with mRNA from the mesothelial cell line 44R.M.-4 treated with carrier substance PBS (lane 1) and crocidolite asbestos at 10 $\mu g/cm^2$ (lane 2), 1 $\mu g/cm^2$ (lane 3), 0.1 $\mu g/cm^2$ (lane 4) or 0.01 $\mu g/cm^2$ (lane 5) suspended in PBS. M, molecular weight marker. The arrow indicates a band of 500 bp. The β-actin gene served as a positive control.

all genes identified by the array assay were expressed in tumors induced by asbestos (lanes 1), whereas no expression was detectable in non-tumorous omenta (lanes 4). For comparison, RT–PCR analysis was also performed with mRNA from tumors induced by nickel powder and benzo[*a*]pyrene in the same experimental system and that are histologically identical to the tumors induced by asbestos (15). In tumors induced by benzo[*a*]pyrene, the expression of all genes was weakly detectable (lanes 3). In nickel-induced tumors, *egfr* and *ilk* expression was detected but not *fra-1* and c-*myc* expression (lanes 2). These data demonstrate that genes expressed in non-tumorous tissue in response to asbestos are also expressed in the asbestos-induced tumors. To some extent, the upregulation of these genes occurs in tumors induced by other environmental carcinogens and may, therefore, be a feature of mesothelioma.

Expression of fra-1 and c-myc can be induced in vitro

Since *fra-1* and c-*myc* are early responsive genes, the next experiment aimed to investigate whether these genes are responsive under *in vitro* conditions. The mesothelial cell line 44R.M.-4, as well as primary mesothelial cells, were treated in culture with 10 $\mu g/cm^2$ crocidolite asbestos for 48 h and mRNA expression was determined by RT–PCR. Figure 3a shows the increase in RT–PCR products of *fra-1* and c-*myc* in primary cells and in the cell line treated with asbestos (lanes 2 and 4). In the controls treated only with carrier solution (lanes 1 and 3), no expression (*fra-1*) or very low expression (c-*myc*) was detected. The genes for *egfr* and *ilk* were not inducible in these experiments (data not shown).

fra-1 expression is dose dependent

The expression of *fra-1* in response to increasing crocidolite doses added to mesothelial cells *in vitro* was measured

by RT–PCR. Figure 3b shows the dose–response of *fra-1* mRNA in the mesothelial cell line 44R.M.-4 treated with 10, 0.1 or 0.01 μg/cm^2 crocidolite asbestos for 48 h. Again, no *fra-1* expression was found in the cells treated with carrier substance. This result demonstrates the responsiveness of *fra-1* mRNA to increasing amounts of crocidolite asbestos *in vitro*.

Discussion

The investigation of asbestos-induced carcinogenesis at the mRNA level revealed a number of genes that were differentially upregulated in certain stages of tumor development. To address whether these genes are also involved in early responses to fiber stress, the expression was investigated using *in vitro* assays with cultured mesothelial cells treated with crocidolite asbestos.

A significant increase in c-*myc* mRNA was detected in pretumorous tissues from asbestos-treated animals, as well as in the late stages of tumor development. Enhanced c-*myc* expression was observed in mesothelial cells treated *in vitro* with crocidolite for 48 h. The gene for cytosolic branched chain amino acid transferase (*bcat*) was also upregulated in fiber-induced mesothelioma. This gene was first described in mice as a target for Myc regulation in c-*myc*-based tumors (16,17). Gene disruption experiments with the homologous *bcat* gene in yeast have revealed correlation with cell cycle regulation at the G_1–S transition (18).

In untransformed cells, Myc expression has been shown to be dependent on mitogenic stimuli and to be required for cell proliferation and to prevent differentiation (19). The Myc–Max heterodimer can bind DNA target sequences regulating genes involved in both cell proliferation and apoptosis (20,21). These two effects occur simultaneously when mesothelial cells are treated with asbestos fibers (22). Our data suggest that, in response to asbestos treatment, cell proliferation and/or apoptosis may be mediated by the c-*myc* proto-oncogene. Similar data come from human tissues investigated at the protein level (23). A significant increase in signal strength in the mesothelioma tissues, compared with basal expression in non-neoplastic mesothelia, has been observed. However, Goodglick *et al.* (24) did not find an increase in c-*myc* mRNA in northern blot analysis with murine mesothelial cell lines derived from different stages of asbestos-induced tumors and reactive tissues. These differing results regarding the expression of the proto-oncogene c-*myc* may demonstrate a species-specific reaction against asbestos in mouse. On the other hand, these differences may be due to the different experimental approaches.

The results from the array assay demonstrated clearly an induction of AP-1 mRNA *in vivo* in fiber-treated tissues. Besides c-*jun*, the *fra-1* gene exhibited enhanced expression at the mRNA level in response to fibers (Table II, Figure 3). The upregulation of this gene in tumors and in fiber-treated cells suggest a role in asbestos-induced carcinogenesis.

Fra-1 forms heterodimers with Jun, generating the AP-1 transcription factor (25). The DNA binding activity and specificity of the Fra-1-containing AP-1 seem to be the same as those of the Fos-containing transcription factor. However, a potent Fos transactivation domain is absent in the smaller Fra-1 protein which, thus, may have a distinct effect. These two characteristics may result in the Fra-1-containing AP-1 having a different specificity from other AP-1 transcription factors.

The dose-dependent induction of c-*jun* and c-*fos* at the transcriptional level in response to crocidolite and chrysotile asbestos in RPM cells has been reported by Heintz *et al.* (26). In hamster tracheal epithelial (HTE) cells, in contrast to c-*jun*, induction of c-*fos* was not detected after treatment with asbestos. The authors suggested that in HTE cells Fra-1 may be the partner for Jun, generating an AP-1 transcription factor that mediates the proliferative effects of fibers (26).

Our results with RPM cells showed that *fra-1* transcription increased, in a dose-dependent manner, after treatment with crocidolite asbestos (Figure 3b). These results indicate that, in asbestos-treated RPM cells, an additional set of genes is activated by the Fra-1-containing AP-1.

The transcription of AP-1 genes in RPM cells in response to fiber treatment is induced by a protein kinase C (PKC)-dependent signal transduction cascade including MAPKs and the extracellular signal-regulated kinases ERK-1 and -2 (27). Interestingly, autophosphorylation of the EGFR, an event activating the ERK cascade, is induced by asbestos fibers (7). In our experiments, enhanced *egfr* expression was detected *in vivo* in pretumorous tissues from asbestos-treated rats, as well as in asbestos-induced tumors. However, there was no increase in *egfr* expression at the mRNA level in asbestos-treated rat mesothelial cells. Thus, *egfr* may not be an early responsive gene. The upregulation in the pretumorous tissues after 52 weeks may result from enhanced receptor turnover during persisting fiber stress. In a human mesothelial cell line, expression and accumulation of the EGFR protein occur *in vitro* after treatment with asbestos fibers (28). However, this upregulation was observed by immunostaining predominantly in singular cells that were in contact with very long fibers ($\geqslant$60 μm) or phagocytosing them. In our *in vitro* experiments analyzing total mRNA from the whole cell population treated with fibers of various length, such an upregulation in individual cells may not be detectable at the mRNA level. Together with the data from mesothelioma cell cultures (4) and from asbestos-treated RPM cells (28), our data suggest that EGFR has a role in fiber-induced mesothelioma development.

A group of genes upregulated by asbestos in the differential RNA analyses is characterized by its connection to integrin-mediated signal transduction. For *opn*, the gene encoding osteopontin, an extracellular glycoprotein, there were higher levels of mRNA in mesothelioma than in non-tumorous tissue. Osteopontin is known to be expressed most prominently in osteoblasts, but enhanced expression has been found in epithelial tissues and in human carcinomas of different origin (29,30). The interaction of osteopontin with several integrins has been reported (31,32). A change in *opn* mRNA expression may suggest signal transduction from this extracellular protein, via integrins, to the intracellular space.

Two genes coding for proteins directly linked to the intracellular part of the focal adhesion contact exhibited elevated mRNA expression. The gene for zyxin (*zyx*) was upregulated in mesothelioma compared with control tissues. Zyxin, a low-abundance phosphoprotein, was discovered by characterization of a rabbit serum that stained subcellular adhesion plaques (33). It consists of three distinct domains: a proline-rich N-terminal region, three double zinc fingers and a central nuclear export signal sequence (34). Both terminal domains interact with structural proteins like α-actinin and various signal transduction proteins. Depending on the stimulus received via the focal adhesion complex, zyxin either acts as

a nuclear shuttle protein or mediates reactions through its influence on cytoskeleton assembly (35).

Integrin-linked protein kinase, another protein directly connected with the focal adhesion contact, was upregulated at the mRNA level *in vivo* in pretumorous asbestos-treated tissues and in mesothelioma (Table II, Figure 2). This enzyme is an intracellular binding partner of integrin β1 (36). In epithelial cell lines, integrin-linked kinase increases the expression of certain cyclines. Overexpression of this protein results in overriding of the adhesion-dependent regulation of the cell cycle progression through G_1 and into S phase, leading to anchorage-independent growth of these cells (37).

Thus, there is upregulation of three independent genes (*opn*, *zyx* and *ilk*) that are linked to transduction of signal from extracellular matrix to the cytoplasm in reaction to asbestos treatment. These results indicate an involvement of integrin-linked signal transduction in asbestos-induced carcinogenesis. Moreover, extracellular matrix proteins seem to be the primary targets of fiber action.

From the results described here, we cannot completely rule out that granular dusts may also have effects on the regulation of the signal transduction genes identified in this study. However, the *in vitro* findings in RPM cells for *egfr* and AP-1 indicate that these effects are specific for carcinogenic fibers (27,28). Moreover, we recently demonstrated at the protein level that Fra-1 can be induced in RPM cells by crocidolite asbestos and man-made fibers (MMVF 21) but not by granulous TiO_2 (38). The physical and chemical properties that are relevant for mRNA upregulation must be investigated further using different fibrous and non-fibrous dusts.

In summary, Fra-*1*, c-*myc* and EGFR may be involved in asbestos-induced mesothelioma carcinogenesis *in vivo*. Our results also suggest that integrin-linked signal transduction pathways and extracellular matrix proteins may be possible targets of fiber effects.

Acknowledgements

The authors wish to thank R.Wirth for technical assistance and Dr R.V.Sorg for reviewing the manuscript.

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Received September 6, 1999; revised January 14, 2000;
accepted January 17, 2000