Mesothelial Cell Transformation Requires Increased AP-1 Binding Activity and ERK-dependent Fra-1 Expression

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ABSTRACT

Mesothelioma is a unique and insidious tumor associated with occupational exposure to asbestos. The transcription factor, activator protein-1 (AP-1) is a major target of asbestos-induced signaling pathways. Here, we demonstrate that asbestos-induced mesothelial cell transformation is linked to increases in AP-1 DNA binding complexes and the AP-1 component, Fra-1. AP-1 binding to DNA was increased dramatically in mesothelioma cell lines in comparison to isolated rat pleural mesothelial (RPM) cells. Elevated levels of AP-1 complexes, including significant increases in c-Jun, JunB and Fra-1, were found in asbestos-exposed RPM cells, but only Fra-1 expression was significantly increased and protracted in both asbestos-exposed RPM cells and mesothelioma cell lines. Asbestos-induced Fra-1 expression in RPM cells was dependent on stimulation of the extracellular signal-regulated kinases (ERKs 1/2). Inhibition of ERK phosphorylation or transfection with dominant-negative fra-1 constructs reversed the transformed phenotype of mesothelioma cells and anchorage-independent growth in soft agar. In summary, we demonstrate that ERK-dependent Fra-1 is elevated in AP-1 complexes in response to asbestos fibers and is critical to the transformation of mesothelial cells.

INTRODUCTION

Inhalation of asbestos in different occupational settings has been associated with the development of pulmonary and pleural disease, primarily asbestosis, lung cancers, and malignant mesothelioma (1). The long latency period of development of malignant mesothelioma (generally 30–40 years) suggests that asbestos fibers may act at several stages of the carcinogenic process, i.e., initiation, promotion, progression. Recent work indicates that asbestos stimulates cell signaling pathways, most notably, the ERK(1) cascade associated with both early injury and subsequent proliferation of mesothelial and pulmonary epithelial cells (2, 3). Asbestos-induced activation of ERKs precedes increases in steady-state mRNA levels of the AP-1 early response proto-oncogenes, c-fos and c-jun, and is accompanied by AP-1 transactivation (4, 5). The dimeric AP-1 transcription factor is composed of members of the Jun, Fos, and activating transcription factor (ATF) families, and the constants of this complex may govern cell proliferation and related responses (6).

In work here, we hypothesized that fra-1, an AP-1 dependent member of the fos family of proto-oncoproteins recently linked to mitogen activation and transformation of epithelial cells (7, 8), is required for asbestos-induced transformation of mesothelial cells through the ERK-MAPK cascade. Here, we demonstrate that mesothelial cell transformation is intimately linked to increases in AP-1 DNA binding complexes and the up-regulation of Fra-1. Our studies also show that

the inhibition of ERK activation or Fra-1 expression abrogates mesothelioma cell growth in soft agar and restores contact inhibition. These observations may be important in preventive and therapeutic approaches to mesothelioma.

MATERIALS AND METHODS

Isolation and Culture of RPM Cells. RPM cells were isolated from the parietal pleura of Fischer 344 rats by methods described previously (4). Cells were used within 10 passages. For all of the experiments, cells were grown to confluence, and 0.5% serum-containing medium was added 24 h before exposure to agents.

Mesothelioma Cell Lines. Mesothelioma cell lines 11, 23, and 52 were derived in rats after peritoneal injection of crocidolite asbestos and have been characterized previously (9). Cells were propagated as described above for RPM cells.

Exposure to Asbestos and Other Agents. Crocidolite asbestos fibers, a high iron-containing amphibole fiber associated with the causation of human mesothelioma (Ref. 1; National Institute of Environmental Health Sciences reference sample) was suspended in HBSS (Life Technologies Inc., Grand Island, NY) at a concentration of 1 mg/ml, triturated eight times through a 22-gauge needle to obtain a homogeneous suspension, and added directly to the medium at a final concentration of 5 μg/cm². EGF (5 ng/ml; Upstate Biotechnology, Lake Placid, NY) was used as a positive control for cell proliferation (3). Glass beads (5 μg/cm²; Particle Information Services, Kingston, WA) were used as a negative control particle. Sham control dishes received medium without agents. The MEK inhibitors, PD98059 and U0126 (Calbiochem) were dissolved in DMSO (0.1% final concentration in medium) and were added to cell cultures at 30 μM and 10 μM, respectively, 1 h before treatment of cells with test agents. U0124 (Calbiochem) at 10 μM was used as a negative control. In these experiments, all of the dishes received 0.1% DMSO in medium (solvent controls).

EMSAs. Electrophoretic gel mobility shift assays (EMSAs) were used to assess the binding of AP-1 to DNA and the composition of AP-1 complexes. Nuclear extracts were prepared and analyzed as described by Janssen et al. (10). The amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) for 15 min at room temperature before the addition of labeled oligonucleotide. Gels were quantitated using a Bio-Rad phosphorimager (Bio-Rad, Hercules, CA).

Western Blot Analyses for Fra-1 and Phosphorylated ERKs. Western blot analyses were used to verify Fra-1 expression in RPM cells exposed to asbestos and to determine whether ERK phosphorylation was blocked specifically by MEK1 inhibitors. Total cell lysates were prepared from control and crocidolite-exposed cells as described previously by Shukla et al. (11). The amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad). Forty μg of protein in sample buffer [62.5 mM Tris-HCL (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.1% w/v bromphenol blue] were electrophoresed in 10% SDS-polyacrylamide gels and transferred to nitrocellulose using a semidry transfer apparatus (Eillard Instrumentation, Ltd., Seattle, WA). Blots were blocked in buffer (TBS containing 5% nonfat powdered milk plus 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) for 1 h, washed three times for 5 min each in TBS/0.5% Tween 20, and incubated for 1 h with specific antibodies (1 μg/ml) to Fra-1, ERK1/2, and p-ERK1/2 (Santa Cruz Biotechnology). Blots were then washed with TBS/0.05%-TWEEN-20, and protein bands were visualized with the LumiGlo enhanced chemiluminescence detection system (Kirkgaard and Perry Laboratories, Gaithersburg, MD).
Transfection Techniques and Constructs. To determine the effect of dnfra-1 and overexpression of fra-1 constructs in mesothelioma and RPM cells, respectively, cells were transfected using electroporation. Briefly, cells were grown to 80–90% confluence, trypsinized, counted, and resuspended at 3 × 10^6 cells/ml at room temperature. An aliquot of the cell suspension (400 μl) was mixed with 10 μg of plasmid DNA (expression or control plasmids) and electroporated at 280 V and 850 μF capacitance. Cells were immediately plated in fresh growth medium in 35-mm culture dishes and allowed to recover overnight. The cells were cotransfected with pNLS-GFP as a marker for efficiency of transfection. GFP detection by fluorescence microscopy showed a transfection efficiency of ~40%. The constructs used in this assay included: (a) pRK7-fra-1, a plasmid that constitutively overexpresses fra-1 from the cytomegalovirus promoter; and (b) a construct in which the leucine zipper responsible for the dimerization function of Fra-1 is deleted (12). (RK7-fra-1Δzip was obtained from Meinrad Busslinger, Research Institute of Molecular Pathology, Vienna, Austria).

Assay for Morphological Transformation. fra-1-transfected cells or cells transfected with dnfra-1 (Δfra-1) were examined for their ability to grow in soft-agar (5). Plates were examined for colony formation, 7 and 14 days later.

Statistical Analyses. In all of the experiments, we used duplicate or triplicate determinations (n = 2–3) per group per time point. Experiments were performed in duplicate. Results were evaluated by one-way ANOVA using the Student-Newman-Keuls procedure for adjustment of multiple pairwise comparisons between treatment groups. Differences with Ps ≤ .05 were considered statistically significant.

RESULTS

AP-1 DNA Binding Activity Is Elevated in Mesothelioma Cell Lines. To determine whether increases in AP-1 activity correlate with transformation of mesothelial cells, we examined AP-1 binding activity in confluent isolated RPM cells and three rat mesothelioma cell lines. As shown in Fig. 1, a significantly elevated (P ≤ 0.05) AP-1 DNA binding activity is observed in confluent rat mesothelioma cell lines developed from asbestos-exposed animals compared with normal RPM cells, indicating that AP-1 transactivation is increased during the process of mesothelial cell transformation.

Asbestos-exposed RPM Cells and Mesothelioma Cell Lines Share an Elevated Fra-1 Component in Their AP-1 Complexes not Present in Normal Mesothelial Cells. To characterize the subunit composition of AP-1 DNA binding complexes in normal RPM cells, asbestos-exposed RPM cells, and mesothelioma cell lines, we used antibodies specific for members of the Fos and Jun families and gel mobility supershift assays. In comparison to untreated RPM cells...
or those exposed to glass beads (GB), significant increases \((P \leq 0.05)\) were found in asbestos-induced c-Jun and JunB at 24 h (Fig. 2). In contrast, no increases in c-Fos, Fra-2, or FosB were seen in asbestos-associated AP-1 complexes. However, striking increases \((P \leq 0.05)\) in Fra-1 were seen in both AP-1 complexes (Fig. 3A) and total cell extracts from asbestos-exposed RPM cells by Western blot analysis (Fig. 3B). In these studies, levels of Fra-1 proteins were increased after 4 h of asbestos exposure and remained elevated at 24 h. c-Jun, Jun B, Jun D, and Fra-2 proteins are components of AP-1 DNA binding complexes in mesothelioma cell lines as well as in confluent, unstimulated RPM cells (Fig. 4). However, AP-1 complexes from mesothelioma cell lines contain Fra-1, which is absent in both confluent (Figs. 3 and 4) and log-phase normal mesothelial cells (data not shown).

**Asbestos-induced Expression of Fra-1 in AP-1 DNA Binding Complexes Is ERK Dependent.** To determine the possible relationship between ERK activation and Fra-1 expression in AP-1 complexes, RPM and mesothelioma cells were pretreated with the MEK1 inhibitor, PD98059, and subsequently exposed to crocidolite asbestos, EGF (5 ng/ml), a positive control inducing cell proliferation, or glass beads for 24 h. As shown in Fig. 5, exposure of RPM cells to asbestos or EGF caused striking increases in levels of Fra-1 proteins in AP-1 DNA binding complexes that were inhibited by pretreatment of cells with PD98059. These results demonstrate that asbestos- and EGF-induced increases in Fra-1 proteins in AP-1 DNA binding complexes are mediated through the MEK1 pathway. No changes in Fra-1 levels were observed in AP-1 complexes from cells exposed to PD98059 alone or to glass beads (negative controls). To confirm that PD98059 was able to block ERK phosphorylation in mesothelioma cell lines 23 and 52, Western blot analysis was performed with antibodies specific for phosphorylated ERKs (p-ERKs). As shown in Fig. 6, treatment of mesothelioma cell lines with the MEK1 inhibitors PD98059 (30 \(\mu\)M) or U0126 (10 \(\mu\)M) produced decreased basal levels of phosphorylated ERK in comparison to untreated cells and cells exposed to the structurally similar, inactive compound, U0124 (10 \(\mu\)M).

**Inhibition of ERK Phosphorylation or Mutated fra-1 in Mesothelioma Cells Causes Reversion to Normal Mesothelial Cell Morphology and Inhibits Their Growth in Soft Agar.** To further demonstrate the role of the ERK pathway in fra-1 transactivation and mesothelioma, we examined the induction of phenotypic changes in RPM and mesothelioma cells exposed to PD98059 (30 \(\mu\)M). PD98059 had a striking and selective effect on the morphology of both of the
evaluated for changes in proliferation and anchorage-independent growth in soft agar. Total cell numbers in RPM cells overexpressing fra-1 or expressing dnfra-1 were not significantly different over time in comparison with cells transfected with empty vector (data not shown). The kinetics of cell proliferation in Meso23 cells transfected with dnfra-1 also were unchanged from vector control groups. On the other hand, dnfra-1-transfected mesothelioma cells showed distinctive changes in cell morphology and decreases in growth in soft agar. Empty vector-transfected mesothelioma cells formed stacked bundles of multilayered spindle cells (Fig. 9A) that flattened and characteristically grew in a single monolayer after transfection with dnfra-1 (Fig. 9B). Mesotheliomas also formed large, spherical colonies when grown in soft agar (Fig. 9C). Colonies decreased in numbers and size after transfection with dnfra-1 (Fig. 9B).

**DISCUSSION**

Malignant mesothelioma is a highly invasive tumor with a poor prognosis, most patients dying within 18 months of initial diagnosis (1). The mechanisms of mesothelial cell transformation by asbestos have been unclear. We show here that asbestos fibers cause selective and protracted increases in the AP-1 family member, Fra-1, a prominent component of the AP-1 complex in asbestos-exposed RPM and mesothelioma cells. Moreover, we demonstrate ERK-dependent induction of Fra-1 and a causal relationship between ERK-activation, Fra-1 in AP-1 complexes, and mesothelial cell transformation. The reversion to a normal morphology and inhibition of anchorage-independent growth in mesothelioma cells transfected with dnfra-1 conclusively demonstrate that Fra-1 is necessary for the maintenance of mesothelial cell transformation. However, because overexpression of fra-1 in normal RPM cells did not result in a transformed phenotype, Fra-1 overexpression alone does not appear to be sufficient for conversion to anchorage-independent growth or loss of contact inhibition.

Little is known about the regulation and functional relevance of Fra-1 in comparison with other members (e.g., c-Jun and c-Fos) of the Fos/Jun family (13). Consistent with our results, overexpression of Fra-1 in nonmalignant 208F or Rat-1 fibroblasts does not cause...
morphological transformation (14). However, expression of exogenous Fra-1 in a noninvasive epithelioid mammary carcinoma cell line results in fibroblastoid conversion and increased invasiveness in vitro (15). These studies and those showing that Rat-1 fibroblasts transfected with fra-1 cause tumor formation in mice (12) suggest that Fra-1 functions in later stages of tumor differentiation and progression. In retrovirally transformed thyroid cell lines, multiple compositional changes in AP-1 exist accompanied by dramatic increases in junB and fra-1 gene expression (8). Moreover, the inhibition of Fra-1 protein synthesis by stable transfection with a fra-1 antisense RNA vector significantly inhibits the malignant phenotype and anchorage-independent growth. In this model, junB is also necessary for the establishment of a fully transformed phenotype. In agreement with our studies in RPM cells, overexpression of fra-1 sense RNA in normal thyroid cells did not cause morphological transformation or growth in soft agar. Our results showing that: (a) transfection of normal mesothelial cells with fra-1 fails to alter cell growth; (b) fra-1 expression is unchanged in actively dividing RPM cells; and (c) dnfra-1 fails to alter the growth kinetics of mesothelioma cells when grown on plastic, suggest that Fra-1 per se does not control cell proliferation. Rather, it appears to influence the conversion of normal epithelial-like cells to a fibroblastoid-transformed morphology acquiring increased anchorage-independent growth.

The sustained expression of Fra-1 in AP-1 complexes in asbestos-exposed mesothelial cells can be compared with studies showing that Fra-1 protein becomes a prominent component of AP-1 complexes after the Ras transformation of NIH 3T3 cells (16). Because inhalation and in vitro exposures to asbestos also cause proliferation of mesothelial cells and elevated mRNA levels of other AP-1 family members, including c-jun, which cause increased proliferation and morphological transformation when overexpressed in tracheal epithelial cells (5), Fra-1 may also cooperate with c-Jun and other Jun family members to induce cell proliferation. These events are critical to tumor promotion and may also render mesothelial cells more susceptible to additional genetic insults. In support of this hypothesis, we show increased Fra-1 in AP-1 complexes of mesothelial cells after the addition of EGF, a known tumor promoter, and others have shown that the tumor promoter, 12-O-tetradecanoyl-13-phorbol acetate (TPA), causes increased Fra-1 in lung cancer cells (17).

Our results and the compendium of studies described above illustrate that Fra-1 induction by asbestos fibers may have multiple roles in the initiation and development of malignancies. The observation that Fra-1 transactivation is ERK dependent may also be relevant to mechanisms of mesothelioma induction by SV40 and the ability of crocidolite asbestos and small t antigen (tag) mutants to complement each other in human mesothelial cell transformation (18). In this regard, SV40tag stimulates ERK activity by binding to and inhibiting protein phosphatase 2A, which dephosphorylates members of the MAPK family (19).

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