

Simian Virus 40 Infection Down-Regulates the Expression of Nitric Oxide Synthase in Human Mesothelial Cells

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Abstract

The cytotoxic effects of asbestos are partly mediated by the production of free radicals, including nitric oxide (NO). SV40 has been suggested to synergize with asbestos in the pathogenesis of malignant mesothelioma. Crocidolite asbestos fibers induced in human mesothelial and malignant mesothelioma cells a significant increase of NO synthase activity and expression, which was absent in SV40-infected cells. Furthermore, SV40 infection prevented the NF κ B activation elicited by crocidolite in both mesothelial and mesothelioma cells. These data suggest that SV40, by inhibiting the synthesis of NO, could favor the survival of transformed, potentially neoplastic cells.

Introduction

Malignant mesothelioma (MM) is a very aggressive neoplasm of the serosal cavities that exhibits a strong correlation with exposure to asbestos fibers (1), such as crocidolite, amosite, and chrysotile. The discovery of SV40 DNA sequences in MM cells has raised the possibility that this virus may act as a cocarcinogen in the development of this cancer (2, 3). The ability of SV40 to inhibit apoptosis, transform cells, and induce tumor formation is dependent on two viral proteins, large tumor antigen (Tag) and small tumor antigen (2). Asbestos fibers cause the production of reactive oxygen species, which are at least partly responsible for the cytotoxic and genotoxic effects of asbestos (1). Cells exposed to asbestos also produce high amounts of the free radical nitric oxide (NO); crocidolite evokes an increased synthesis of NO in rat and murine macrophages, human lung epithelial cells, and rat mesothelial cells (4). NO is synthesized by three NO synthase (NOS; EC 1.14.13.39) isoforms, which favor the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (5); in oxygenated living systems, NO is rapidly converted into nitrite and nitrate (6). NO is highly cytotoxic: Huge amounts of this radical are produced due to activation of the inducible NOS (iNOS) isoform in macrophages and other inflammatory cells, contributing to the flogistic response (5). The nuclear factor κ B (NF κ B) mediates the iNOS induction elicited by lipopolysaccharide and cytokines (5, 7), and the exposure of hamster tracheal epithelial cells, rat lung epithelial and mesothelial cells to crocidolite has been associated with NF κ B activation (1). Our study has been aimed to investigate whether SV40 modulates the crocidolite-stimulated NOS activity and expression, both in human mesothelial cells (HMC) and MM cells

(MMC), and whether the NF κ B signaling pathway is involved in this effect.

Materials and Methods

Reagents. Fetal bovine serum and Ham's F-10 medium were supplied by Life Technologies (Paisley, Scotland); L-[2,3,4,5-³H]arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, United Kingdom). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). The protein content of cell monolayers and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL). If not otherwise specified, other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Aldrich (Milan, Italy).

Cells. Primary MMC cultures and primary HMCs used in the present study have already been characterized (8). MMCs were separated from the pleural effusion of three patients with histologically confirmed MM, one of them SV40 positive (8), whereas HMCs were isolated from three patients with transudative pleural fluid due to congestive heart failure and with no history of malignant disease. The mesothelial origin of the isolated cells was confirmed by positive immunostaining as described previously (8). Cells were cultured in Ham's F-10 nutrient mixture medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-glutamine and maintained in a humidified atmosphere at 37°C and 5% CO₂.

Transfection with SV40 and Tag DNAs. Primary HMC cultures were transfected either with SV40 full-length DNA or with the recombinant vector pSV3neo expressing Tag by Prof. Mauro Tognon (Department of Morphology and Embryology, University of Ferrara, Italy). Nontransfected HMCs were used as controls. Stable cell cultures were examined by immunoblotting using a monoclonal antibody to Tag (Ab-1; Oncogene Science, Tarrytown, NY).

Asbestos Fibers. Union International Contre le Cancer crocidolite fibers were sonicated (Labsonic sonicator; 100 W; 10 s) before incubation with cell cultures to dissociate fiber bundles and allow their better suspension and diffusion in the culture medium. To set the fiber concentration, we checked the production of nitrite (see next paragraph) in the presence of different amounts of fibers (1, 5, and 25 μ g/cm²) in both HMC and MMC cultures after 24-h and 48-h incubation times. In parallel, we measured the release of lactate dehydrogenase as an index of cytotoxicity. On the basis of these preliminary results (data not shown), we chose to incubate the cells with 25 μ g/cm² crocidolite for 24 h, because this was the most effective dose and time condition still allowing an acceptable survival of mesothelial cells (about 45% dead HMCs or MMCs after 24 h).

Measurement of NOS Activity and Nitrite Production. Cells grown at confluence on 35-mm diameter Petri dishes after a 24-h incubation with crocidolite fibers were lysed, and NOS activity was checked as the ability of the cell lysates to convert L-[³H]arginine to [³H]citrulline during 15 min, as described previously (9). The enzyme activity was expressed as pmol of citrulline/min/mg of cell protein. In parallel, the nitrite concentration in the culture medium was measured with the Griess method, as described previously (9), and expressed as nmol of nitrite/24 h/mg of cell protein.

Western Blot. Whole-cell extracts containing equal amounts of proteins (30 μ g) were probed with anti-inhibitor of nuclear factor- κ B (I κ B)- α antibodies (from rabbit, diluted 1:200 in PBS-BSA 1%; Santa Cruz Biotechnology, DBA, Italy), as described previously (9).

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Reverse Transcriptase-PCR. Reverse transcriptase-PCR from total RNA was carried out using the prepared cDNA as a template, as described previously (9). The same cDNA preparation was used for reverse transcriptase-PCR of iNOS and β -actin, checked as a housekeeping gene.

Electrophoretic Mobility Shift Assay (EMSA). Cells were plated in 100-mm diameter dishes at confluence, and all procedures for nuclear protein extraction and EMSA were performed as described previously (9).

Statistical Analysis. Each experimental point has been performed in duplicate or triplicate per experiment; all data in the text and figures are given as means \pm SE. The results were analyzed by a one-way ANOVA and Tukey's test. $P < 0.05$ was considered significant.

Results and Discussion

HMCs and MMCs exhibited a basal NOS activity, measured as the ability of the cell lysate to convert L-[3 H]arginine to L-[3 H]citrulline and a detectable level of nitrite (a stable derivative of NO oxidation) in the culture supernatants (Fig. 1). After a 24-h incubation with crocidolite, HMCs and MMCs showed a significant increase of intracellular NOS activity and extracellular nitrite level; such an increase was absent in HMCs transfected with full-length SV40 or Tag and in SV40-containing MMCs (Fig. 1). In reverse transcriptase-PCR experiments, under the same conditions crocidolite increased the amount of iNOS mRNA in HMCs and MMCs, but this effect was completely abolished in the presence of SV40 (Fig. 2). β -Actin expression was not significantly different in the experimental conditions investigated (Fig. 2).

The mechanism of NOS stimulation by asbestos fibers is not completely clear: Asbestos has been suggested to induce NOS expres-

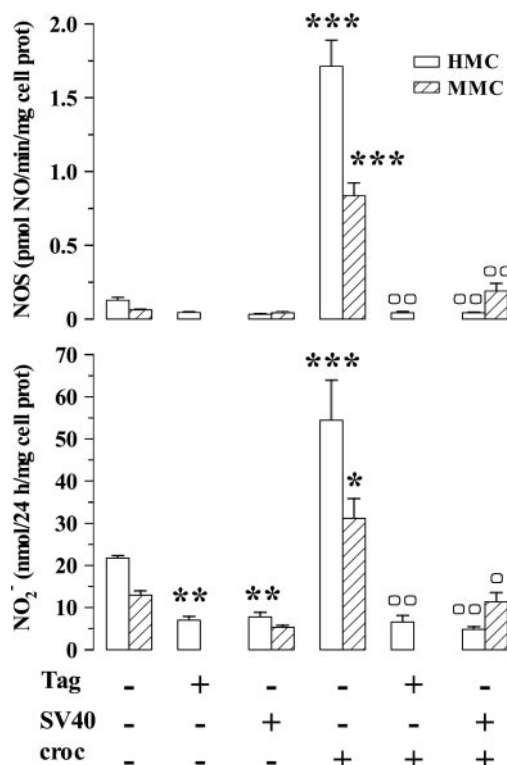


Fig. 1. NOS activity (top panel) and nitrite levels (bottom panel) in HMCs (\square), in the absence (-) or presence (+) of Tag or SV40 full-length DNA, and in MMCs (▨), negative (-) or positive (+) for SV40. Cells were incubated for 24 h in the absence (-) or presence (+) of crocidolite fibers (croc; 25 $\mu\text{g}/\text{cm}^2$). After this time, NOS activity was measured in the cell lysates (containing 100 $\mu\text{g}/100 \mu\text{l}$ cell proteins), and extracellular medium was checked for nitrite concentration (see "Materials and Methods"). Measurements were performed in duplicate, and data are presented as means \pm SE ($n = 6$). Versus respective HMC or MMC control (Tag -, SV40 -, croc -): ***, $P < 0.0001$; **, $P < 0.003$; *, $P < 0.05$. Versus respective crocidolite treatment (Tag -, SV40 -, croc +): \square , $P < 0.0001$; \square , $P < 0.05$.

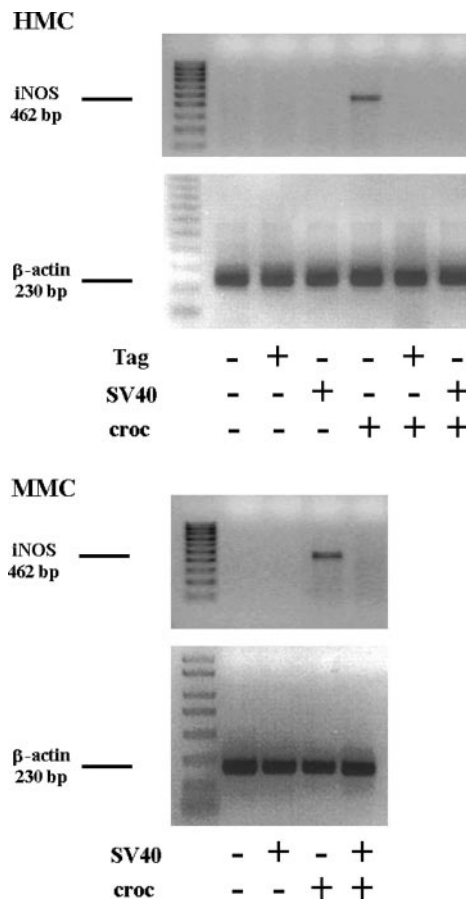


Fig. 2. Reverse transcriptase-PCR of iNOS mRNA in HMCs (top panel), in the absence (-) or presence (+) of Tag or SV40 full-length DNA, and in MMCs (bottom panel), negative (-) or positive (+) for SV40. Before RNA extraction, cells were incubated for 24 h in the absence (-) or presence (+) of crocidolite fibers (croc; 25 $\mu\text{g}/\text{cm}^2$). The reverse transcriptase-PCR efficiency was controlled by amplifying a β -actin fragment, used as a housekeeping gene. Representative figure of three similar experiments.

sion by evoking an iron-mediated oxidative stress and/or stimulating an oxidative burst in phagocytes (4). An increased production of reactive oxygen species has been demonstrated to activate the redox-sensitive transcription factor $\text{NF}\kappa\text{B}$, which is known to bind to the iNOS promoter (5), and asbestos has been observed to cause nuclear translocation of $\text{NF}\kappa\text{B}$ in rat lung epithelial and pleural mesothelial cells (10). Till now, asbestos has been shown to induce NO synthesis in mesothelium, but only in interleukin-1 β -stimulated rat mesothelial cells (11). To our knowledge, this is the first study showing that the treatment with crocidolite asbestos fibers induces a significant increase of NOS activity and nitrite production also in HMCs (from both normal pleura and MM). Unlike rat mesothelial cells, human cells exhibited a crocidolite-induced increase of NOS activity without needing a prestimulation with cytokines.

To clarify the mechanism by which SV40 prevents the iNOS gene transcription, we investigated the role of $\text{NF}\kappa\text{B}$. The EMSA experiments, performed in nuclear extracts of both HMCs and MMCs, showed that $\text{NF}\kappa\text{B}$, after a 24-h stimulation with crocidolite, translocated into the nucleus; whereas in the presence of SV40, this effect was inhibited (Fig. 3). $\text{NF}\kappa\text{B}$ designates a family of proteins assembled in dimeric transcription factors that are involved in the activation of a large number of genes, in response to a wide range of infectious diseases and cellular stresses (7). In unstimulated cells, the nuclear localization of $\text{NF}\kappa\text{B}$ is hindered by the binding of the inhibitory protein $\text{I}\kappa\text{B}$, which sequesters $\text{NF}\kappa\text{B}$ in the cytoplasm. Cellular stimulation with inflammatory cytokines, bacterial lipopolysaccharide, or

potent oxidants results in I κ B-kinase-mediated phosphorylation, ubiquitination, and proteasomal degradation of I κ B (7). Western blotting analysis showed that the cytosolic level of I κ B α was significantly reduced in crocidolite-treated cells but not when cells contained SV40 (Fig. 4). These results suggest that SV40 prevents the crocidolite-induced proteolytic degradation of I κ B α protein and the consequent activation and translocation of NF κ B, thus accounting for the suppression of iNOS gene expression and NO synthesis in cells stimulated with crocidolite. The mechanism of this SV40-mediated I κ B stabilization is currently under investigation in our laboratory.

Asbestos has been observed to induce both proliferation and apoptosis in many cell types (4), including mesothelial cells (12). If the growth arrest or apoptotic responses to asbestos-induced DNA damage are evaded, a multistep accumulation of genetic abnormalities may occur, favoring the evolution to mesothelioma (13). Mutations or inhibition of the tumor suppressors protein p53 may be a mechanism by which cells bypass programmed cell death (13). Because mesotheliomas, unlike most tumors, do not exhibit p53 mutations (14), it is conceivable that the Tag-mediated inhibition of p53 by SV40 infection or some growth factor overriding the p53 signal is more important in favoring asbestos carcinogenesis (2, 13).

It is known that asbestos fibers cause cytotoxic effects and mutagenic changes through the production of free radicals such as reactive oxygen species and NO (1, 4). NO can react with the superoxide anion, producing either peroxynitrite, which can generate hydroxyl radicals, or other reactive nitrogen species able to nitrate proteins and nucleic acids (6). By inducing the synthesis of reactive oxygen and nitrogen species, asbestos fibers exert a cytotoxic effect that favors the

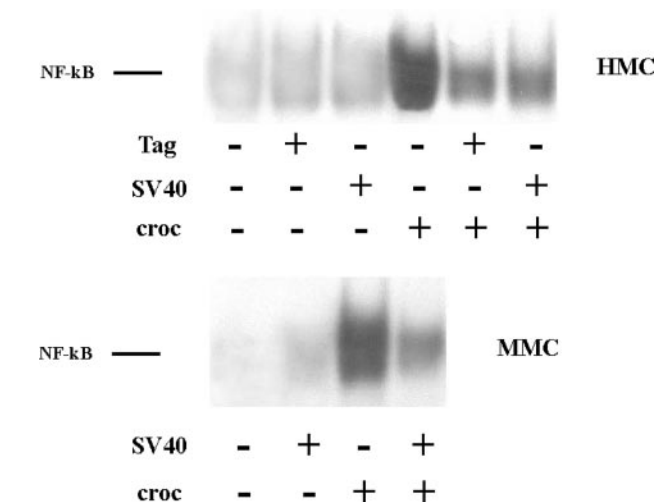


Fig. 3. EMSA detection of NF κ B activation in HMCs (top panel), in the absence (-) or presence (+) of Tag or SV40 full-length DNA, and in MMCs (bottom panel), negative (-) or positive (+) for SV40. Cells were incubated for 24 h in the absence (-) or presence (+) of crocidolite fibers (croc; 25 μ g/cm²). Nuclear extracts were subjected to EMSA as described in "Materials and Methods." Representative figure of three similar experiments.

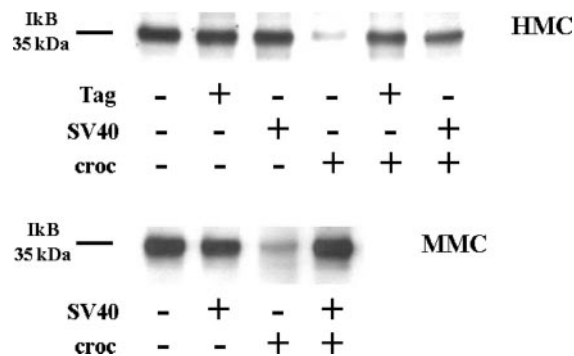


Fig. 4. Western blot detection of I κ B α in HMCs (top panel), in the absence (-) or presence (+) of Tag or SV40 full-length DNA, and in MMCs (bottom panel), negative (-) or positive (+) for SV40. Cells were incubated for 24 h in the absence (-) or presence (+) of crocidolite fibers (croc; 25 μ g/cm²). Representative figure of three similar experiments.

elimination of damaged cells. Our results suggest that the inhibition of NO synthesis following SV40 infection could be one of the mechanisms leading to a decreased apoptosis and allowing the survival of transformed and potentially neoplastic cells.

Acknowledgments

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