A Mutant Epidermal Growth Factor Receptor Targeted to Lung Epithelium Inhibits Asbestos-induced Proliferation and Proto-Oncogene Expression

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Abstract

Asbestos is a ubiquitous naturally occurring fiber causing multiple cancers and fibroproliferative disease. The mechanisms of epithelial cell hyperplasia, a hallmark of the initiation of lung cancers by asbestos, have been unclear. We demonstrate here that mice expressing a dominant-negative mutant epidermal growth factor receptor (EGFR) under the control of the human lung surfactant protein-C promoter exhibit decreased pulmonary epithelial cell proliferation without alterations in asbestos-induced inflammation. In contrast to transgene-negative littermates, inhalation of asbestos by mice expressing the mutant EGFR does not result in early and elevated expression of early response proto-oncogenes (fos/jun or activator protein 1 family members). Additionally, quantitative reverse transcriptase-PCR analysis for levels of c-jun and c-fos in bronchiolar epithelium isolated by laser capture microdissection demonstrates increases in expression of these genes in asbestos-exposed epithelial cells. Results show that the EGFR mediates both asbestos-induced proto-oncogene expression and epithelial cell proliferation, providing a rationale for modification of its phosphorylation in preventive and therapeutic approaches to lung cancers and mesothelioma.

Introduction

Asbestos is a ubiquitous family of naturally occurring fibrous minerals used in thousands of commercial products around the world. Chrysotile asbestos [white asbestos, Mg₆Si₄O₁₀(OH)₈], the most common type of asbestos used historically in industry and crocidolite asbestos [blue asbestos, (Na₂(Fe³⁺)₂(Fe²⁺)₃Si₂O₇(OH))₂], a fiber which may be more potent in the development of mesothelioma and lung cancers (1, 2), are two of the most widely studied forms of asbestos fibers. Occupational exposures to asbestos are associated with numerous pleuropulmonary disorders and diseases, including lung cancer (3, 4). The increased incidence of asbestos-associated cancers and asbestosis is of critical concern as therapies for these diseases are limited and their prognosis dismal.

After chronic or lifetime inhalation of asbestos fibers, rodents develop few mesotheliomas or lung cancers but die of asbestosis, which develops in parallel with hyperplastic and neoplastic epithelial changes. Cell proliferation after exposure to asbestos fibers is a consequence of early asbestos-induced injury to pulmonary epithelial and mesothelial cells and is critical to the development of malignancies and other fibroproliferative diseases (3, 4). Asbestos fibers cause aggregation and increased immunoreactivity of the EGFR protein in human mesothelial cells in vitro (5), and these events may initiate signaling pathways important in asbestos-induced proliferation and carcinogenesis. In both mesothelial and alveolar type II epithelial cells, EGF and asbestos stimulate DNA synthesis (6, 7). Moreover, phosphorylation of the EGFR by asbestos fibers is causally related to increases in phosphorylation and activity of ERKs 1/2 (8, 9), mitogen-activated protein kinases that are increased at sites of asbestos deposition, and proliferation in lungs after inhalation of fibers (10). Asbestos fibers also cause elevations in EGFR mRNA and protein (9), indicating their potential roles in both EGFR phosphorylation and biosynthesis.

Another consequence of asbestos exposure in mesothelial and pulmonary epithelial cells is the induction of the AP-1 family members, c-fos and c-jun, and increases in AP-1 activity (11, 12). The AP-1 early response proto-oncogenes are linked to proliferation and transformation in a number of cell types, representing one common pathway by which cells respond to environmental stress. An understanding of their role in the pathogenic responses to asbestos provides insight into the mechanisms of asbestos-related diseases, as well as the broader role that these genes play in cellular proliferation, transformation, and apoptosis in a number of cell types.

Our studies here were designed to determine whether activation of the EGFR is critical to cell proliferation and proto-oncogene expression induced by asbestos fibers in pulmonary epithelial cells, cell types initially encountering and interacting with asbestos fibers after inhalation, as well as target cells affected in lung cancers and asbestosis. Because asbestos fibers are deposited after inhalation in the distal lung, primarily in the alveolar duct region, we used transgenic mice expressing a mutant EGFR (Tg⁻⁻) lacking a portion of the intracytoplasmic phosphorylation domain and targeted to alveolar epithelial cells, EGF and asbestos stimulate DNA synthesis (6, 7). Moreover, phosphorylation of the EGFR by asbestos fibers is causally related to increases in phosphorylation and activity of ERKs 1/2 (8, 9), mitogen-activated protein kinases that are increased at sites of asbestos deposition, and proliferation in lungs after inhalation of fibers (10). Asbestos fibers also cause elevations in EGFR mRNA and protein (9), indicating their potential roles in both EGFR phosphorylation and biosynthesis.

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3 The abbreviations used: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; AP-1, activator protein 1; Tg⁻⁻, transgene negative; Tg⁺⁺, transgene positive; SP-C, surfactant protein C; BAL, bronchoalveolar lavage; QRT-PCR, quantitative reverse transcriptase-PCR; 6-FAM, 6-carboxyl-fluorescein; TAMRA, 6-carboxyl-tetramethyl rhodamine; LCM, laser capture microdissection; RPA, ribonuclease protection assay; TGF-α, transforming growth factor α.
Materials and Methods

Transgenic Mice. Breeding pairs of the 9527 line were obtained from Dr. Thomas Korfhagen (Children’s Hospital, Cincinnati, OH). These mice express a mutant EGFR lacking a portion of the intracytoplasmic phosphorylation domain (deleted for sequences beyond amino acid position 714) under control of the lung-specific human SP-C promoter (13). In situ hybridization showed that transcripts of the transgene were selectively expressed in distal bronchiolar and alveolar type II epithelial cells (13). Progeny were crossed with 2–3-month-old C57/BL6 mice (an average of 4 mice/group/time period) to generate littermates that were used in subsequent experiments. In Experiment 1, both Tg^+ and Tg^- mice (an average of 4 mice/group/time period) were evaluated for proliferative responses to asbestos after 2- and 4-day exposures. Sham mice were killed at 3 days. Forty-eight and 24 h before killing, both sham and asbestos-exposed mice received i.p. injections of BrdUrd (100 mg/kg body weight) as described previously (15) to allow a direct comparison between numbers of proliferating epithelial cells detected by the BrdUrd and PCNA techniques. Because trends were comparable, the PCNA method, a less invasive method not requiring preinjection of animals before death, was used in subsequent experiments. In Experiment 2, inflammation was characterized in sham and asbestos-exposed C57/BL6 mice at 1, 3, and 9 days (n = 8/group/time period). In Experiment 3, both Tg^+ and Tg^- mice (n = 8/group/time period) were examined for proliferation and inflammation at time periods demonstrating changes, i.e., 4 and 9 days, in Experiments 1 and 2, respectively, and at 30 days. Mice were given a lethal i.p. dose of pentobarbital and evaluated as described below.

Histopathology. After opening the chest cavity, a polyurethane catheter was inserted into the trachea and the right lung lobes sutured, removed, placed in a cryomold, and immersed in optimal cutting temperature (OCT) embedding compound (Tissue Tek; Sakura Finetek USA, Torrance, CA) before being snap-frozen in liquid nitrogen-cooled 2-methylbutanane. Left lung lobes were then instilled with 4% paraformaldehyde at a pressure of 25 cm of water, allowed to fix for 5 min, and placed in a tissue cassette overnight in 4% paraformaldehyde at 4°C before embedding in paraffin blocks. Lung sections were cut at a thickness of 4 μm for histochemistry and histopathology after staining using the Masson’s trichrome technique.

Immunohistochemistry for PCNA. Lung sections from sham and asbestos-exposed mice were deparaffinized three times in xylene for 5 min, rehydrated through a series of graded alcohols, and equilibrated with PBS. Slides were then permeabilized with 100% methanol for 10 min at −20°C followed by a 15-min incubation with 0.1% Triton-X100. After rinsing with PBS, the slides were washed with 10 ml citrate buffer, then boiled for 10 min. Tissue sections were incubated in 200 ml of goat serum in 10 ml of 0.1% Triton-X100 in PBS three times for 20 min before addition of a biotinylated antibody to PCNA (PharMingen, San Diego, CA; 1:500 dilution). Lung sections were then incubated with streptavidin followed by a brief incubation in 3,3′-diaminobenzidine as a chromogen, according to the manufacturer’s protocol (Vector Laboratories, Burlingame, CA). Sections were then rinsed with distilled H2O, counterstained with hematoxylin, dehydrated through a series of graded alcohols, cleared with xylene, and mounted using Vectashield (Vector Laboratories).

Image Analysis. Numbers of epithelial cells incorporating nuclear PCNA in the distal bronchiolus and alveolar duct regions were quantitated using image analysis. In brief, immunostained sections were imaged through a BX-50 Olympus microscope (Olympus Corp., Tokyo, Japan). Images were collected with a color video camera and adapter coupled with an Olympus viewing screen and remote control unit (Sony Corp., Tokyo, Japan). Images were stored and analyzed using a SunSPARC Station 5 computer (Sun Microsystems, Mountain View, CA) using Imaris/Imagist Version 8 Integrated Microanalyzer for imaging software (Princeton Gamma-tech, Princeton, NJ). Images were then converted from RAF to TIFF files and analyzed using Paint Shop Pro 3 and Optimas photo programs. Using a blind coding system, the number of PCNA-positive epithelial cells in distal bronchiolus and the alveolar duct region was assessed independently by two individuals by tracing the unit length of basal lamina of each of these compartments and expressing results per unit length as described previously (14). A total of 10 discrete structures was evaluated on individual lung sections (n = 2/mouse).

BAL. After the chest cavity was opened, a polyurethane catheter was placed in the trachea, and lungs were lavaged in situ six times with a 1-ml volume of sterile calcium and magnesium-free PBS at 37°C. BAL samples from each mouse were pooled, measured, and centrifuged to obtain cell pellets for total and differential cell counts. Total cell counts were determined using a hemocytometer. Cytospin preparations were made as described previously (14, 15), and slides were stained with Giemsa and May Grunwald stains. Five hundred cells were evaluated on each slide.

Cell Cultures. The C10 cell line is a nontumorigenic murine alveolar type II epithelial cell line originally derived from a NAL 1A alveolar type II epithelial cell line (16). The cell line was isolated from adult mice and maintains a characteristic epithelial cell morphology, including surface microvilli, desmosomes, and lamellar bodies. C10 cells were maintained and passaged in Connaught Medical Research Laboratories 1066 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mm l-glutamine, and antibiotics. At confluence, cells were switched to 0.5% serum-containing medium for 24 h before addition of crocidolite asbestos (National Institute of Environmental Health Sciences reference sample; Research Triangle Park, NC). Fibers were weighed after sterilization in a dry oven to remove possible endotoxin and so on and suspended at 1 mg/ml in Hank’s Balanced Salt Solution before trituration in a syringe and addition at 5 μg/cm² area of dish, a concentration-inducing cell proliferation (7). AG1478 (10 μM), an inhibitor of EGFR phosphorylation (8, 9), and the MAP/ERK kinase 1/2 inhibitors PD98059 (30 μM; Ref. 7) or U0126 (10 μM; all from Calbiochem, San Diego, CA) were added alone or 30 min before asbestos in 0.1% DMSO-containing medium. Solvent controls and initial studies with the structurally similar, inactive compound U0124, which had no effects on ERK activation or proto-oncogene expression, were also included.

RPA. Total RNA was prepared from C10 cells and lung homogenates as described previously (17). Steady-state mRNA levels of c-jun, jun-B, jun-D, c-fos, fra-1, fra-2, fosB, L32, and glyceraldehyde-3-phosphate dehydrogenase were examined using the RiboQuant multiprobe RPA system and the Mfos/Fun multiprobe template set (PharMingen), according to the manufacturer’s protocol. Autoradiograms were quantitated using a Bio-Rad phosphorimager. Results were normalized to expression of the housekeeping gene L32.

LCM. Distal lung bronchiolar epithelial cells were microdissected from 10-μm thick cryostat sections cut from mouse lungs frozen in OCT. The sections were cut at −23°C with stainless steel blades mounted in a Microm Micromet Cryostate and picked up onto Fisherbrand Superfrost/Plus-treated slides (Fisher Scientific, Pittsburgh, PA). The sections were stored at −80°C until use. Sections were fixed for 30 s in 70% ethanol, rinsed with autoclaved-double-distilled H2O for 30 s, and dehydrated with 70, 95, and 100% ethanol. To ensure dehydration, slides were rinsed in fresh xylenes twice for 10 min. The dehydrated sections were stored in a dessicator until use. All sections were used within 24 h of dehydration. Microdissection was performed with an Arcturus PixCell II LCM system (Arcturus Engineering, Mountain View, CA), according to the manufacturer’s protocol. After microdissection, debris that had adhered nonspecifically to the CapSure film was removed using a CapSure Pad (Arcturus Engineering).

QRT-PCR. After RNA isolation from the CapSure film using the PicoPure RNA isolation kit according to manufacturer’s instructions, RNA was treated with RQI RNase-free DNase (Promega, Madison, WI), at a concentration of 0.1units/μl, to remove genomic DNA. Using the isolated RNA as a template, cDNA was prepared using the Promega Reverse Transcription kit, according to manufacturer’s instructions. Using a Perkin-Elmer ABI 7700 Prism Sequence Detection System (Applied Biosystems, Foster City, CA), the relative abundance of cDNA corresponding to c-jun, c-fos, and hpit were quantitated relative to the levels in standard curves generated from C10 cells treated with 12-O-tetradecanoylphorbol-13-acetate for 2 h. Levels of c-jun and c-fos were then normalized to levels of hpit to allow comparisons between samples. Probes for QRT-PCR analysis used 6-FAM as a fluorophore and either TAMRA or Black Hole Quencher-1 (BHQ-1) as a quencher on the 3’-end. The forward (GAAAACCTTGAAAGCGCAAAAC) and reverse (CACCTGTTCGACATGCATG) primers and probe (6-FAM-CCGAGCGCTCATCGGACATGCTCAATC),
and probe for hprt (6-FAM-CGACCCGAGTCCAGGGTC-BHQ-1) were purchased from Biosearch Technologies (Novato, CA). The forward primer for c-fos (AAATCCAGGGCCACAGGA), reverse primer for c-fos (CCGACCTGACGCAGAT), and probe for c-fos (6-FAM-TCTCTCTGGGAAGGCCAGGTCATCG-TAMRA) were also purchased from Biosearch Technologies. Probes for all three genes were used at a concentration of 200 nM. Primers were used at a concentration of 900 nM.

Statistical Analysis. All data are presented as means ± SE. Statistical significance was evaluated by ANOVA using a Student Neuman-Keuls test. \( P \leq 0.05 \) were considered statistically significant.

Results

Epithelial Cell Proliferation Is Increased after Inhalation of Asbestos and Inhibited in Tg\(^+\) Mice Expressing a Mutant EGFR.

In comparison to sham controls, mice exposed to asbestos exhibited increases in numbers of PCNA-positive epithelial cells in distal bronchioles and the alveolar duct regions of the lung. Significant increases \( (P \leq 0.05) \) in proliferation were first observed after 4 days exposure to asbestos in Tg\(^-\) but not Tg\(^+\) mice in both these compartments (Fig. 1). Moreover, significantly fewer \( (P \leq 0.05) \) PCNA-positive epithelial cells were noted in Tg\(^-\) versus Tg\(^+\) mice at 4 days in the distal bronchioles (Fig. 1a), and only Tg\(^-\) mice exposed to asbestos showed significant \( (P \leq 0.5) \) elevations in proliferation of alveolar epithelial cells when compared with Tg\(^-\) sham mice (Fig. 1b). At 30 days, asbestos-exposed Tg\(^+\) and Tg\(^-\) mice showed increased numbers of PCNA-positive epithelial cells in both compartments of the lung in comparison to sham controls (Fig. 1c,b). However, numbers of alveolar type II epithelial cells showing positivity were decreased in Tg\(^+\) in comparison to Tg\(^-\) mice \( (P \leq 0.05) \) in the alveolar duct region (Fig. 1d). After inhalation and deposition, asbestos fibers are transported distally throughout the lung, a process that may reflect the more striking earlier bronchiolar changes and later responses in the alveolar compartment. Immunohistochemistry for PCNA (Fig. 2, a and b) and histopathology verified that bronchiolar and epithelial cell hyperplasia, as well as inflammation, occurred in Tg\(^-\) asbestos-exposed mice (Fig. 2d) in comparison to lungs of unexposed animals (Fig. 2c). Tg\(^+\) unexposed lungs were normal (Fig. 2e), whereas Tg\(^+\) lungs exposed to asbestos exhibited inflammatory foci (Fig. 2f).

Pulmonary Inflammation by Asbestos Is Not Modified in Tg\(^+\) Mice Expressing a Mutant EGFR. BAL was used as a sensitive and quantitative technique to characterize the inflammatory responses of
mice exposed to asbestos. We determined previously that total cell numbers in BAL were elevated \((P < 0.05)\) in mice after 3 and 9 days exposure to asbestos (10). This was also reflected by increases in the proportions of neutrophils at 3 and 9 days and of lymphocytes at 9 days. On the basis of these data, we examined patterns of inflammation at 9 days in Tg/ and Tg/ animals. Both total cell counts and proportions of macrophages, lymphocytes, and neutrophils in BAL were similar in both sham and asbestos-exposed Tg/ and Tg/ mice (Fig. 1, e and f), supporting the histopathology observed in these lungs. The fact that inflammation is comparable in both Tg/ and Tg/ asbestos-exposed animals suggests that inflammatory cytokines or chemokines do not play a direct role in acute epithelial cell proliferation.

The Induction of \(fos\) and \(jun\) Family Proto-Oncogenes Is Altered by Disruption of Signaling through EGFR. In comparison to sham controls, analysis of lung tissue homogenates of Tg/ mice by RPAs (see representative autoradiograph in Fig. 4a) showed significant up-regulation \((P \leq 0.05)\) of \(fos\) and \(jun\) family members after 4 days of exposure to asbestos but no elevations at the 30-day time point, consistent with an early response (Fig. 1, e and f). Lungs of Tg/ mice showed no elevations in \(fos\) and \(jun\) family members after inhalation of asbestos at either time point. QRT-PCR results from bronchiolar epithelium obtained by LCM show that c-\(fos\) and c-\(jun\) message levels were increased specifically in these cell types in C57/BL6 mice exposed to asbestos.

To confirm a direct link between EGFR phosphorylation and increased expression of early response proto-oncogenes, pulmonary epithelial cells were exposed to asbestos alone and after preaddition of AG1478. This inhibitor of EGFR phosphorylation significantly diminished \((P < 0.05)\) asbestos-induced elevations in \(jun\)-B and \(fra\)-1 mRNA levels (Fig. 4). Expression of \(jun\)-B, c-\(fos\) and \(fra\)-1 by asbestos was also abrogated with use of the ERK inhibitors PD98059 and U0126 but not U0124 (data not shown); these observations support our prior work showing that ERK activation by asbestos is modulated through the EGFR (7, 8).

Discussion

Data here are the first to show that asbestos-induced epithelial cell mitogenesis \textit{in vivo} is mediated in part via phosphorylation of the EGFR. Genetically modifying the EGFR to prevent its phosphorylation abrogates acute (4 day) cell proliferation in both epithelial compartments of the lung, \textit{i.e.}, distal bronchiolar epithelium and alveolar type II epithelium, expressing the transgene. At 30 days, significant decreases in epithelial cell proliferation were still observed in Tg/ type II epithelial cells in the alveolar duct region, but labeling in Tg/ and Tg/ distal bronchiolar epithelial cells was comparable. These temporal patterns may reflect the fact that increases in other cytokines (platelet-derived growth factor and so on) stimulating MAPK cascades and other proliferation, and survival pathways are produced in response to asbestos (4). Results are noteworthy as they are the first to show that asbestos fibers must interact with and phosphorylate a growth factor receptor to cause proliferation, a phenotypic alteration key to the development of malignant and fibroproliferative lung
diseases. For example, epithelial hyperplasia and squamous metaplasia are well-recognized lesions in the spectrum of premalignant changes leading to lung cancers and are observed commonly in the respiratory tract of smokers and asbestos workers (18).

Findings here may also be relevant to the development of mesothelioma and fibrosis as links between EGF, TGF-α, which binds to the EGFR, and cell proliferation have been demonstrated in mesothelioma cells (19). Moreover, patients with asbestosis, in comparison to control individuals, show increased amounts of the extracellular domain of the EGFR in sera (20). Previous work has also demonstrated that immunoreactivity of EGFR and TGF-α is increased after bleomycin-induced epithelial lung injury and pulmonary fibrosis (21). Overexpression of TGF-α targeted to lung epithelium using the human SP-C promoter causes the development of fibrotic lesions in transgenic mice (22) that can be reversed in bi-transgenic TGF-α overexpressing mice crossbred with mice expressing a mutant EGFR (13). TGF-α null mutant mice also are resistant to bleomycin-induced pulmonary fibrosis and do not exhibit compensatory increases in other EGF family members in lung (23). The demonstration that TGF-α immunoreactivity is increased in pulmonary epithelium after brief inhalation of asbestos fibers (24) also suggests that asbestos may cause biosynthesis of TGF-α which then may become available to cause phosphorylation of the EGFR. In addition, studies in our laboratory have shown that asbestos exposure in vitro induces increased EGFR mRNA and protein synthesis in mesothelial cells (9). Recent studies also suggest that growth factors such as platelet-derived growth factor or EGF may act in concert to cause phosphorylation events critical in mitogenesis of lung myofibroblasts and fibrogenesis (25).

We document here a temporal pattern of AP-1 family member proto-oncogene expression in the lungs of mice exposed to asbestos. The acute elevations at 4 but not 30 days are consistent with an early response and may be connected causally with cell proliferation. Numerous studies have indicated the involvement of AP-1 in the initiation of mitogenesis and transformation (reviewed in Ref. 26).

Fig. 3. RPAs (a) showing steady-state mRNA levels of fos/jun family members in lung homogenates of Tg− and Tg+ sham control and asbestos-exposed mice. Note the striking increase in mRNA levels of fos/jun family members at 4 days in Tg− mice. QRT-PCR (b) from bronchiolar epithelium obtained by LCM. Expression levels of c-jun and c-fos are normalized to hprt expression. * = P ≤ 0.05 in comparison to respective Tg− and Tg+ sham control groups at each time point.
Whereas c-Fos and c-Jun have been studied extensively, less is known about other members of the Fos/Jun family, including Fra-1, an AP-1 dependent gene as well as a prominent component of AP-1 complexes in C-10 (17) and mesothelial cells exposed to asbestos.

A role for protracted Fra-1 expression in eliciting S-phase increases in response to silica or asbestos has been implicated in recent work (17). Moreover, ERK-dependent Fra-1 transactivation has recently been linked to mitogenesis and tumor promotion in mouse epidermal cells (27). Previous studies have also shown that c-fos is induced in an EGFR-dependent manner in mesothelial cells in vitro in response to asbestos (9). It is noteworthy that results here are the first to show that EGFR phosphorylation plays a critical role in inducing fos/jun proto-oncogenes in both pulmonary epithelial cells in vitro and in an inhalation model of asbestos-induced proliferation. This work is also novel in that LCM and QRT-PCR are used to demonstrate that increased expression of AP-1 family members occurs specifically in asbestos-exposed bronchiolar epithelial cells.

Our results suggest a model for induction of asbestos-induced proliferation via phosphorylation of the EGFR. In this scheme, EGFR phosphorylation leads to activation of ERK 1/2. ERK 1/2 activation, in turn, leads to increased fos/jun family member expression and incorporation of the corresponding proteins into AP-1 complexes. Subsequent AP-1 activation then favors cell cycle progression and proliferation of epithelial cells. The resulting hyperplasia may then contribute to the development of lung cancers and mesothelioma. More importantly, our work shows that asbestos-induced epithelial cell proliferation can be prevented by inhibiting phosphorylation of the EGFR, a finding that may be applicable to control of epithelial cell responses to asbestos and other environmental insults, including oxidants and UV irradiation, which phosphorylate the EGFR (28–30).

Given the fact that inhibitors of EGFR function are already in advanced stages of clinical trials, linking the inhibition of EGFR function with the prevention of progression of asbestos-related diseases may point to a possible therapeutic strategy for these diseases (31).

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References


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