Asbestos induction of extended lifespan in normal human mesothelial cells: interindividual susceptibility and SV40 T antigen

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Normal human mesothelial cells from individual donors were studied for susceptibility to asbestos-induction of apoptosis and generation of an extended lifespan population. Such populations were generated after death of the majority of cells and arose from a subset of mesothelial cultures (4/16) whereas fibroblastic cells (5/5) did not develop extended lifespan populations after asbestos exposure. All mesothelial cultures were examined for the presence of SV40 T antigen to obtain information on (i) the presence of SV40 T antigen expression in normal human mesothelial cells and (ii) the relationship between generation of an extended lifespan population and expression of SV40 T antigen. Immunostaining for SV40 T antigen was positive in 2/38 normal human mesothelial cultures. These cultures also had elevated p53 expression. However, the two isolates expressing SV40 T antigen did not exhibit enhanced proliferative potential or develop an extended lifespan population. Asbestos-generated extended lifespan populations were specifically resistant to asbestos-mediated but not α-Fas-induced apoptosis. Deletion of p16INK4a was shown in 70% of tumor samples. All mesothelioma cell lines examined showed homozygous deletion of this locus which extended to exon 1B. Extended lifespan cultures were examined for expression of p16INK4a to establish whether deletion was an early response to asbestos exposure. During their rapid growth phase, extended lifespan cultures showed decreased expression of p16INK4a relative to untreated cultures, but methylation was not observed, and p16INK4a expression became elevated when cells entered culture crisis. These data extend the earlier observation that asbestos can generate extended lifespan populations, providing data on frequency and cell type specificity. In addition, this report shows that generation of such populations does not require expression of SV40 T antigen. Extended lifespan cells could represent a population expressing early changes critical for mesothelioma development. Further study of these populations could identify such changes.

Introduction

Mesothelioma is a form of cancer in which exposure to asbestos, a complete carcinogen, produces sufficient alterations in normal mesothelial cells to generate a malignant population (1,2). These changes arise, presumably, both by direct interaction with asbestos fibers (3–5) and by the generation, in response to asbestos, of an inflammatory environment containing strong proliferative signals as well as active oxygen species (6–8). Interestingly, mesothelioma cell lines and tumors have been shown to contain few p53 mutations (9–11) and a normal retinoblastoma (RB) gene product (12–14). Since the functions of these tumor suppressor genes are inactivated in most malignancies, mesothelioma is a tumor that is likely to contain genetic or epigenetic alterations in molecules which interfere with p53 and/or RB function. Recent reports have associated the presence of SV40 T antigen sequences and protein with mesothelioma tissue (15,16). Clearly, the presence of a molecule such as T antigen, which could inactivate both p53 and RB, would explain the lack of alterations of these suppressors.

Homzygous deletion of sequences on chromosome 9p21–22 is associated with mesothelioma (17–19) and loss or reduction of p16INK4a has been demonstrated in this tumor (20). Loss or inactivation of this gene has been shown to diminish RB cell cycle control, and loss of p16INK4a and alternation of RB have been shown to have a reciprocal relationship (12,21,22). In addition, it has been shown that the alternate reading frame protein, p14ARF, interacts with MDM2 (23,24) to affect the stability of the protein and is transcriptionally activated by E2F-1, linking release of this factor by RB phosphorylation to p53 stabilization (25). Thus, loss of the two functions of this locus in mesothelioma could reduce the activity of both p53 and RB.

Mesothelial cells exposed to asbestos show responses ranging from a proliferative response (26–29) to cell cycle arrest or apoptosis (30–32). These responses are likely to be mediated by altered gene expression following induction of transcription factors and activation of intracellular signaling pathways (33–35). Interestingly, early studies of in vitro amosite treatment of normal human mesothelial cells demonstrated the establishment of a population of cells with an extended lifespan and chromosomal alterations (36,37). Identification of molecular alterations in such a population might reveal changes that occur before immortalization, which are critical in the process of tumorigenesis. Epigenetic down-regulation of p16INK4a has been shown in extended lifespan populations of mammary epithelial cells (38,39). Data from studies on bladder cancer specimens indicate that progression to myoinvasive tumors correlates with alteration of RB or p16INK4a and the ability to bypass senescence in culture (40).
Thus, alteration of p16^{INK4a} expression might contribute to the extended lifespan induced by asbestos exposure. In addition, expression of SV40 T antigen, if present in the normal population, would be expected to increase the probability of expressing an extended lifespan (41,42). Therefore, measurement of SV40 T in the mesothelial isolates tested for lifespan extension should provide data concerning the biological relevance of this gene to mesothelial carcinogenesis.

The data presented here confirm that direct in vitro treatment of normal human mesothelial cells with amosite asbestos induces an acute apoptotic response, which is followed, in some cases, by generation of an extended lifespan population. While all mesothelial isolates showed an apoptotic response, only a subset of isolates generated extended lifespan populations. These biologically altered populations were characterized by a decreased sensitivity to asbestos-induced apoptosis but not to apoptosis mediated by Fas ligand-binding. SV40 T antigen was expressed infrequently in normal human mesothelial cells with amosite asbestos exposure.

About 25 000 cells per well were inoculated in six-well dishes. After asbestos or exposure to 200 ng/ml anti-Fas (45) (anti-Fas, clone CH-11, Medical and Biological Laboratories, Boston, MA), both detached and attached cells were collected and concentrated by centrifugation. The medium was removed and the cells were resuspended in 100 µl of 1× binding buffer (1 ml of 1 M HEPES, pH 7.0 in 95 ml of distilled water, pH adjusted to 7.4, after which 2.8 ml of 5 M NaCl, 0.037 g CaCl2 dihydrate and distilled water were added to a total volume of 100 ml). Annexin-V-FITC (final concentration 11 ng/ml) (Bio Whittaker, Walkersville, MD) and propidium iodide (PI, final concentration 2.5 µg/ml) were then added. Following incubation at room temperature in the dark for 5 min, 20 µl of cell suspension was placed on a glass slide and a cover slip applied. These slides were evaluated by fluorescence microscopy. At least 200 cells were counted in each group. Cells with annexin V-positive staining only were scored as early apoptosis. Cells with both positive staining of annexin V and PI and with condensed nuclei were counted as late apoptosis (32). Cells exhibiting PI staining without condensed nuclei were not scored as apoptotic (Figure 2).

Immuno-fluorescent staining

Cells on glass slides were fixed with 4% paraformaldehyde [in phosphate-buffered saline (PBS)] for 10 min at room temperature followed by absolute methanol for 20 min at room temperature. Slides were washed three times with PBS-plus solution (0.15 g glycine, 0.5% BSA in 100 ml PBS) before incubation with primary antibodies at 4°C overnight (16 h) then at room temperature for 30 min. Primary antibodies were used at the following concentrations: p16^{INK4a} (Ab-1), 2.5 µg/ml; p21 (WAF1, Ab-1), 2.5 µg/ml; p53 (Ab-6), 1.0 µg/ml; and SV40 T-Ag (Ab-2, N-terminal), all from Oncogene Research Products (Cambridge, MA), 0.2 µg/ml; SV40 T-Ag (Pab101, C-terminal), Santa Cruz Biotechnology (Santa Cruz, CA), 0.2 µg/ml. After five washes with PBS, slides were incubated with goat anti-mouse-fluorescein isothiocyanate (FITC) (1:300) (Vector Lab, Burlingame, CA) for 1 h at room temperature and washed again with PBS, five times. A drop of VectaShield, (Vector Lab), which contained 0.5 µg/ml 4′-6-diamidino-2-phenylindole (DAPI), was added; cover slips were applied and results were evaluated by fluorescence microscopy. The percentage of positive cells was determined by counting at least 200 cells. For determination of staining intensity, 100 positive cells were scored using the segmentation program of the fluorescent imaging system (IPLab Spectrum®32, Version 3.1, Scanalytics, Fairfax, VA).

PCR and methylation specific PCR

Genomic DNA from cultured cells was prepared using DNAzol (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer’s instruction. For standard PCR amplification of p16^{INK4a}, the primers for exon 2 were used: sense, 5′-ATGGGGCGGAGGGGTGATCCG-3′ and antisense, 5′-TTATCTGTACCTTGTGCC-3′. PCR reactions contained, in 50 µl, genomic DNA 200 ng, 1× PCR buffer, 1.1 mM Mg(OAc)2, 0.2 mM dNTP, 15 pmol each primer and 1 U (Th.DNA polymerase, XL, PE Applied Biosystems, Norwalk, CT). Reaction conditions were 94°C, 4 min, then 94°C for 40 s, 60°C for 30 s, 68°C for 2 min 30 s, for 35 cycles followed by 68°C for 8 min. For methylation specific PCR, DNA (1 µg) in a volume of 50 µl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. An aliquot of 30 µl of 10 mM hydroquinone (Sigma, St Louis, MO) and 520 µl of 1 M NaOH was added. Samples were mixed, and samples were kept under mineral oil at 50°C for 16 h. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega, Madison, WI) and eluted into 50 µl of water. Modification was completed by NaOH (final = 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was resuspended in 50 µl water and used immediately or stored at −20°C (46). Primers used for a first amplification of p16 exon 1 outside the potential methylated region were p16 wild-type, sense 5′-CAAGGAGGGGGGGGACCCG-3′, antisense 5′-CGGCGCGCGCGCGTG-3′ (size, 140 bp; annealing temperature 65°C; genomic position +171); p16 methylation specific, sense 5′-TTATAGAAGTGGGGGGCGTCCG-3′, antisense 5′-GACCGGCAACC-GGACCGCTA-3′ (size, 150 bp; annealing temperature 65°C; genomic position +167); and p16 unmethylated specific, sense 5′-TTATAGAAGTGGGGGGCGTCCG-3 ′, antisense 5′-AAAAACCCCAACCCACCAATAA-3′ (size, 151 bp; annealing temperature 60°C; genomic position +167). The PCR mixture contained 1× PCR buffer (16.6 mM ammonium sulfate, 67 mM...
Tris, pH 8.8, 6.7 mM MgCl₂, 10 mM 2-mercaptoethanol), dNTP (each at 1.25 mM), primers (300 ng each per reaction) and bisulfite-modified DNA (~50 ng) or unmodified DNA (50–100 ng) in a final volume of 50 µl. PCR specific for unmodified DNA also included 5% dimethyl sulfoxide. Reactions were subjected to a hot start at 95°C for 5 min before the addition of 1.25 U Taq polymerase (BRL) followed by 35 cycles (30 s at 95°C, 30 s at the annealing temperature listed above, and 30 s at 72°C) and a final 4 min extension at 72°C. Products were analyzed on 2% agarose gels.

**Southern blot**

DNA was extracted, digested with HindIII, probed with 32p-labeled p16^INK4a and XBP probes and analyzed according to standard protocols (47). Ratios of p16^INK4a to XBP were determined for each sample and normal and tumor samples were compared to determine the relative content of p16^INK4a in each sample.

**Western blot**

Whole cell lysates were made by lysing cells in RIPA buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% deoxycholic acid, plus the following protease inhibitors added at time of use from the stock solutions: 10 µl/ml of 10 mg/ml polyethylene sulfonide fluoride (PMSF) in isopropanol, 30 µl/ml of aprotonin (Sigma) and 10 µl/ml of 100 mM sodium orthovanadate]. Lysates were centrifuged at 40 000 r.p.m. for 35 min at 4°C (Sorvall RC-5B ultracentrifuge in an RFP24-AT-80 rotor). Aliquots of 50 µg protein/sample were loaded on a pre-cast 16% Tris–glycine gel (Novex, San Diego, CA) run at 125 V for ~3 h until the running dye was eluted and then transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked for 2 h at room temperature in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), which contained 5% non-fat milk and 0.5% BSA, incubated with anti-p16 antibody [polyclonal antiserum, cat. # 15126E, 1:1000 dilution in blocking solution, PharMingen (San Diego, CA)] at 4°C for 16 h; washed, and incubated for 1 h with a 1:10 000 dilution of anti-rabbit horseradish peroxidase antibody (NA 934, Amersham, Arlington Heights, IL) in blocking solution at room temperature; washed and developed with enhanced chemiluminescence (ECL) solution (34080, Pierce, Rockford, IL) according to the manufacturer’s instructions.

**Results**

**Comparative toxicity of amosite preparations for mesothelial cells and fibroblasts**

Recent reports show either apoptosis (31,32) or cell cycle arrest (30) induced by asbestos treatment of mesothelial cells and suggest that these may have arisen because of differences in fiber types used by different laboratories (30,48). We compared two types of amosite with different fiber dimensions, UICC and NAIMA (see Materials and methods) with respect to toxicity in normal human mesothelial cells in order to establish the dose range for amosite treatment. Fibroblasts were also exposed to the NAIMA amosite to establish cell-type dose responsiveness. Figure 1 shows the results of a 72 h treatment of either mesothelial cells or fibroblasts. Four individual cell strains of each cell type were tested. Interest-ingly, the toxicity curves did not show significant inter-individual variation in contradistinction to the growth potential variation shown by mesothelial cells from different individuals (49). It is apparent, in confirmation of earlier reports using colony-forming assays (36), that human mesothelial cells show greater sensitivity to asbestos toxicity than do fibroblasts. The data of Figure 1 indicate that the NAIMA amosite, selected for longer fibers is, as expected, more toxic than UICC amosite with an LD₅₀ of 2.4 µg/cm² for mesothelial cells as compared with 10.8 µg/cm² for fibroblasts.

**Induction of apoptosis in normal human mesothelial cells by amosite asbestos**

The induction of apoptosis in normal human mesothelial cells by increasing doses of UICC or NAIMA amosite was evaluated after a 24 h exposure. Both types of amosite-induced apoptosis at levels in general agreement with the observations of Broaddus et al. on normal human mesothelial cells (32), suggesting that the fiber length may not account for the observation of cell cycle arrest rather than apoptosis as a response to asbestos exposure (30,48). Figure 2a illustrates the appearance of cells scored for early and late apoptosis. In Figure 2a-A, the two cells designated by arrows are examples of early apoptosis, staining for annexin but not internalizing PI. In contrast, in Figure 2a-C and a-D, the two indicated cells are examples of late apoptosis with annexin staining and PI showing condensed or fragmented nuclei. The cell on the upper right is negative for apoptosis. The apoptotic response to amosite was studied as a function of dose and time (Figure 2b). Exposure to glass beads did not induce apoptosis above the basal level of 5% (data not shown). The data of Figure 2b-A indicate that induction of apoptosis by NAIMA amosite was greater than by UICC amosite across the dose range tested. Study of the time-dependence of the apoptotic response to NAIMA amosite showed that apoptotic cells were still increasing at 48 h (Figure 2b-B). However, at 36 h, apoptotic cells showed comparable numbers of early and late apoptotic changes. By 48 h, cells exhibiting late apoptosis represented the increasing fraction whereas the percentage of early apoptosis was level, indicating that some cells were continuing to initiate the process of apoptosis.

**Extended proliferative lifespan cultures of human mesothelial cells generated by asbestos treatment**

Mesothelial isolates grown from pleural fluids were selected for asbestos treatment based on growth potential. Cultures were initially seeded at a ratio of 1 T75 flask to 100 ml of pleural fluid. Although a precise count of the original cell number was not possible, because the pleural fluid contains macrophages and red blood cells that are not eliminated until the first passage, we estimate that 15–20 population doublings occurred before cells reached 80% confluence at this original passage. Cells that required >1 month to reach 80% confluence were not selected for asbestos treatment. A total of 16 mesothelial and five fibroblastic isolates were treated with the protocol described in Materials and methods, which repeated that used earlier (36) except that the asbestos dose was reduced to LD₅₀ from LD₉₀. Interestingly, when some cell strains were tested at multiple doses, doses below LD₅₀ did not produce populations exhibiting an extended proliferative lifespan (data not shown). Figure 3A (mesothelial strain 15244) shows a growth curve typical of that shown by all four mesothelial isolates that exhibit an extended proliferative lifespan after asbestos treatment (Table I). The observation that four of 16

![Fig. 1. Cytotoxicity of amosite for human mesothelial cells and fibroblasts.](image-url)
isolates treated exhibited this pattern suggests that generation of such cultures by asbestos treatment is not a rare event. Furthermore, cells from two isolates exhibiting extended proliferative lifespans were frozen before asbestos treatment, thawed, and treated with amosite to test the reproducibility of the phenomenon. These cells again generated an extended proliferative lifespan. However, cells from all four extended lifespan cultures ceased dividing and reached a static period of culture crisis. Interestingly, none of the five individual fibroblastic isolates tested showed an increase in lifespan after asbestos treatment. In fact, as shown in Figure 3B, all fibroblastic isolates whether treated with glass beads (▲) or amosite (■) showed growth inhibition, relative to the mock-treated controls. This comparison between mesothelial cells and fibroblasts

Table I. Normal human mesothelial cell strains. Donor, T antigen and experimental status

<table>
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Consecutive samples of normal human mesothelial cells were propagated from non-malignant pleural fluids and characterized with respect to SV40 T antigen expression, proliferation potential and response to asbestos treatment as detailed in Materials and methods.
Extended lifespan in asbestos-treated human cells

Extended proliferative lifespan populations (ELP) or mock-treated populations (Mock) were compared with MeT-5A, a T antigen-immortalized, non-tumorigenic human mesothelial cell line or M9K, a human mesothelioma cell line with respect to induction of apoptosis after 24 h by antibody to Fas or by 12.5 μg/cm² NAIMA amosite.

Extended proliferative lifespan cultures of human mesothelial cells generated by asbestos treatment are resistant to asbestos-induced apoptosis

If asbestos treatment selects or creates a sub-population of mesothelial cells with an enhanced proliferative potential, this population should be resistant to asbestos-induced cell death. To test this prediction, extended proliferative lifespan populations from all four positive donors were exposed to different doses of NAIMA amosite and scored for apoptosis by antibody to Fas protein and viable cell number after 24 h. The graphs of Figure 4 combine data obtained from all isolates and demonstrate the decrease in induction of apoptosis by amosite in the population exhibiting an extended proliferative lifespan as compared with the mock-treated cell controls (Figure 4A). When viable cells were counted, however, the difference in asbestos sensitivity between mock-treated (LD₅₀ = 4.0) and extended lifespan (LD₅₀ = 7.1) was less marked (Figure 4B).

Extended proliferative lifespan populations (ELP) were indistinguishable (Figure 4C) and exhibited 40.5 ± 16 or 40 ± 13% apoptotic cells, respectively, after 24 h. In all cases, 60% of cells were dead by 48 h and 90–100% were dead by 72 h. Interestingly, the T-antigen immortalized, non-tumorigenic cell line MeT-5A (41) showed a similar degree of apoptosis (33 ± 3.6%) to these normal mesothelial cell strains when treated with anti-Fas, whereas the p53 wt mesothelioma cell line, M9K (9), was resistant even to anti-Fas generated apoptosis (15 ± 2.6%). When exposed to 12.5 μg/cm² amosite, however, these two cell lines were equally resistant (MeT-5A = 12 ± 2 and M9K = 7.5 ± 0.9% apoptosis). This observation suggests that additional molecular alterations accumulated in mesothelioma cells during carcinogenesis affect apoptosis at steps common to multiple induction pathways, which is in agreement with the broad apoptotic resistance demonstrated by mesothelioma cell lines (50).

Selection of genes for analysis in extended proliferative lifespan cultures

The observation that only four of 16 mesothelial isolates generated an extended proliferative lifespan population suggests that some individuals are more likely than others to show this response to asbestos. Such a response may reflect a higher probability of developing mesothelioma if this population represents an early stage in carcinogenesis. It is reasonable to assume that, in particular, genes associated with senescence or limited lifespan might be inactivated.

SV40 large T antigen

Recent reports demonstrate the presence of SV40 large T antigen DNA sequences (reviewed in 51) and protein (15,16) in human malignant mesothelioma specimens and suggest that these represent viral infections introduced with SV40-contaminated lots of polio vaccine. We reasoned that, if individuals had been infected, their normal cells might show evidence of this infection. Furthermore, presence of large T antigen would be expected to increase the probability that a human mesothelial cell strain would exhibit an extended proliferative lifespan in tissue culture (41). We therefore analyzed all normal human mesothelial isolates without regard to proliferative potential for the presence of T antigen by immunohistochemistry. A total of 38 sequential specimens were analyzed, of which two exhibited positive staining for T antigen. In both T positive specimens, as in MeT-5A, the p53 wt mesothelioma cell line, M9K (9), was resistant to asbestos treatment. However, this sample did not generate an extended proliferative lifespan.

Importance of p16INK4a loss in mesothelioma

Previous reports (17–20,22) indicate that homozygous deletion of the p16INK4a gene is very common in mesothelioma samples. In addition, re-expression of p16INK4a in mesothelioma cells and xenografts has been shown to inhibit growth and tumorigenicity.

![Graphs](https://academic.oup.com/carcin/article-abstract/20/5/773/2529734/105x58)
Since high level expression of this gene has been shown to correlate with senescence, and decreased expression has been observed in mammary epithelial cells that escape the M0 growth arrest characteristic of the first in vitro phase of primary mammary epithelial cell culture (34,38), we examined matched normal and tumor DNA samples for the frequency of gene deletion in tumors. Southern blotting of DNA samples probed sequentially for p16Ink4a and for XPB as a constitutive control indicated that loss of p16Ink4a relative to XPB had occurred in 75% (15/20) of the samples tested (Fig. 6, Table II). Since values for tumor samples reflect stromal contamination, these values suggest that the 11 samples with ratios below 0.5 (Table II) are likely to be homozygously deleted. Additional analysis of normal, tumor and cell line DNAs from individuals whose tumors had yielded mesothelioma cell lines tested earlier for p53 status (20 cell lines) (9), were tested for presence of the p15ink4b, p16ink4a and p14ARF genes by amplifying the second exon of p15ink4b and p16ink4a as well as exon 1β from p14ARF. In agreement with earlier observations showing deletion of this region in mesothelioma (18), all cell lines were negative for amplification from these primers whereas normal human mesothelial cells and the T antigen immortalized Met-5A were positive. Positive amplification from β-globin primers established the integrity of the mesothelioma sample DNA (data not shown). These data support the conclusion that the entire region of chromosome 9p from p15ink4b through p16ink4a is homozygously deleted in these cell lines and that expression of an active N-terminal fragment of ARF does not occur.
Ink4a expression can be detected by western blotting of for expression of the encoded protein. Figure 8 shows that non-tumorigenic mesothelial cell lines expressing T antigen expression is significantly less than that seen in immortalized, related to the proliferative potential of the culture. As expected, expression level is variable among cell isolates and could be µ50.

As previously mentioned, p16Ink4a, at least at the GC island analyzed. evidence of silencing by methylation prior to deletion of expression in Figure 7A show that normal samples revealed DNA methylation (samples 3, 4, 8, 9 and 22) more frequently than tumor samples (samples 7 and 8). Of the 19 samples analyzed, seven normal and four tumor samples showed methylated product. No sample showed only a methylated band. Extended proliferative lifespan cells at pd20 and mock-treated controls at population doublings 4 (mock) or 20 (asbestos-treated). As shown in Figure 9A and B, the percentage of positive cells as well as the intensity of p16Ink4a expression decreased during the rapid proliferative period of the extended proliferative lifespan but increased again when the cells ceased growing.

Expression of p16Ink4a in mesothelial cells after asbestos exposure

Since deletion of the p16Ink4a region is common in mesothelioma, we examined tumor material that showed p16Ink4a bands on Southern analysis (Figure 6, Table II) for p16Ink4a methylation to examine the possibility that methylation precedes deletion. Methylation sensitive primers, as described in Materials and methods, were designed for amplification of sequences for p16Ink4a exon1. All p16Ink4a positive tumor and normal DNAs used for Southern blotting were treated as described in Materials and methods and amplified first with primers outside the GC-rich region and then with primers designed to amplify methyl-protected or unprotected residues in the 174–185 GC island. MeT-5A and Colo320 were used as negative and positive controls for methylation. The results in Figure 7A show that normal samples revealed DNA methylation (samples 3, 4, 8, 9 and 22) more frequently than tumor samples (samples 7 and 8). Of the 19 samples analyzed, seven normal and four tumor samples showed methylated product. No sample showed only a methylated band. Extended proliferative lifespan cells at pd20 and mock-treated controls at population doublings 4 (mock) or 20 (asbestos-treated). As shown in Figure 9A and B, the percentage of positive cells as well as the intensity of p16Ink4a expression decreased during the rapid proliferative period of the extended proliferative lifespan but increased again when the cells ceased growing.

Expression of p53 and p21 after asbestos exposure

Since the ELP represents cells that have escaped and are resistant to asbestos induction of apoptosis, it was of interest to examine whether p53 or p21 expression levels were normal or decreased relative to control mesothelial cells. Immunohistochemical staining of these populations in comparison with mock-treated proliferating cells showed similar steady state levels of both genes in asbestos-treated and control cells (Figure 10).

Discussion

Recent data have demonstrated the presence, in human mesothelioma, of SV40 T antigen sequences and protein, and have suggested that T antigen may play a role in the genesis of mesothelioma through binding to and inhibiting the tumor suppressors p53 and RB (51). These findings suggest the hypothesis that SV40, present in normal human mesothelial cells, might contribute to tumorigenesis by enhancing the proliferative potential of these cells as well as increasing DNA instability. The tumor types that demonstrate SV40 T sequences might have cellular precursors that are selective reservoirs for the virus. Treatment of normal human mesothelial cells with asbestos has been shown previously to select or create cells that continue to proliferate longer than untreated controls and demonstrate chromosomal abnormalities (36). Generation of extended lifespan populations in human cells is associated with inactivation of the p53 protein and/or the pRB gene family through mutation or loss of these genes or their regulators or by expression of DNA virus oncoproteins (42). We examined the 38 human mesothelial strains utilized in this study for the expression of SV40 T antigen by immunohistochemistry. Two isolates were positive for both N- and C-
Fig. 9. Expression of p16INK4a in extended lifespan and mock-treated normal human mesothelial cells. (A) Cells from various growth phases of mesothelial strains 15244, 14928 and 11936 were studied for expression of p16INK4a by immunocytochemistry as described in Materials and methods. The percentage of positive cells (% positive cells) was determined by counting at least 200 cells after staining for p16INK4a as described in Materials and methods. The staining intensity of p16INK4a (% staining intensity) was determined by segmentation analysis. The data shown in A combine data obtained from three mesothelial isolates and error bars represent standard deviation of the mean values. (B) Expression of p16INK4a in mock- and asbestos-treated strain 15244 mesothelial cells was analyzed as described for A by immunocytochemistry. Population doublings were determined as described in Materials and methods. As shown in Figure 3A, mock-treated cells ceased dividing at pd5 whereas cells exhibiting proliferative lifespan extension were dividing actively at pd20 but ceased dividing at pd30.

Fig. 10. Expression of p53 and p21WAF1 in an extended lifespan population. Extended lifespan- (pd20) and mock-treated (pd4) cultures were analyzed as described in Materials and methods for expression of p21 and p53.

terminal epitopes and showed the presence of elevated p53 expression as would be expected from stabilization of the wild-type protein in complex with T antigen (Table I; Figure 5A and B). The fulfillment of these criteria suggests that functional T antigen was expressed in these two mesothelial cell strains. Interestingly, the two T antigen positive isolates were at least 95% positive for expression of the protein and of p53. This observation contrasts with the staining of ≤50% in T antigen positive mesothelioma cells in culture (15) and suggests that T antigen function may not be critical after tumorigenic conversion but does raise the issue of what mechanism might account for a partial loss in a tumor of clonal origin (55). The data available (age and sex) for the two donors of T antigen positive cells do not distinguish them from other donors in this study, although it is relevant that their ages (69 and 76), like the age of most donors sampled (Table I), make it possible that they received contaminated polio vaccines as adults (56). However, although the small number of samples makes this evidence anecdotal, the low proliferation rate of one strain and the failure of the other to generate an extended lifespan population suggest that the T antigen expressed in these cells did not significantly contribute to an enhanced proliferative potential. An alternative possibility, given the great variability of lifespan and growth potential among normal mesothelial cell isolates (49), is that the proliferative lifespan of the T antigen positive cells was, in fact, extended beyond that which would have been observed in the absence of T antigen.

Acute responses of mesothelial cells to asbestos treatment have been difficult to relate to mechanisms of carcinogenesis. This report confirms the generation, by in vitro asbestos treatment of normal human mesothelial cells, of populations of cells that survive the initial apoptotic response to exhibit an extended proliferative lifespan (36,37). Such a population would represent a target population of altered mesothelial cells, which would be expected to demonstrate resistance to asbestos-induced cytotoxicity (57). It is our hypothesis that these cells have lost ‘gatekeeper’ or ‘caretaker’ functions (58) and are primed for further alterations contributing to tumorigenicity. In this study, normal human mesothelial and fibroblastic cell strains from individual donors were treated with amosite asbestos. After documentation of the acute apoptotic and necrotic death of these cells, culture was continued both to examine the frequency with which fiber exposure generated a population of cells with an extended lifespan and to evaluate molecular characteristics of such populations.

Amosite asbestos samples of different fiber length (UICC and NAIMA) were compared for induction of cell death and apoptosis (Figures 1 and 2). As expected, the NAIMA sample containing longer fibers generated higher levels of cell death and apoptosis per dose. Levels of apoptosis observed in response to amosite were in general agreement with those reported for crocidolite or amosite in other studies (31,32), which suggests that fiber length alone does not explain the observation of cell cycle arrest as opposed to apoptosis after asbestos treatment (30).

Mesothelial cells were exposed to amosite according to a previous protocol that had yielded extended lifespan cells (36) but at doses (2.4 or 4.8 µg/cm²) close to the LD₉₀ determined for this lot of fibers (Figure 1) instead of LD₃₀. Sixteen mesothelial strains were exposed to asbestos (Table I) and four of these generated a population that exhibited an extended proliferative lifespan. Mock-treated or glass bead-treated cul-
tures of these strains exhibited lower proliferation rates and entered culture crisis within another five population doublings (Figure 3). These data indicate that asbestos treatment generates or selects a subset of cells from a normal human mesothelial population. The observation that only four of 16 isolates generated such populations suggests the presence of interindividual differences that predispose a cell strain to exhibit this asbestos response. Differentiated characteristics may be important in this response since none of the five individual fibroblastic isolates treated developed an extended lifespan (Figure 3B). It is logical to predict that a population generated or selected by asbestos exposure should be resistant to asbestos-generated cytotoxicity and apoptosis. This report demonstrates that these cells, which are apoptosis competent when stimulated by antibody to Fas (Figure 4C), are specifically resistant to induction of apoptosis by asbestos exposure (Figure 4A). This resistant population expresses both p53 and p21 at the levels seen in untreated cells (Figure 10). In addition, it has been shown that many mesothelioma cell lines lacking p16INK4a contain wild-type p53, which is elevated in response to γ-irradiation and can induce p21 transcription (59).

The data of Figure 4 show that resistance to death by apoptosis (Figure 4A) accounts for the resistance shown by this population to asbestos cytotoxicity (Figure 4B). We have shown earlier that human mesothelioma cell lines are resistant to apoptosis in response to asbestos and calcium ionophore. This resistance does not arise from high expression of Bcl-2, which was detected in only 3/14 mesothelioma lysates in contrast to the pro-apoptotic Bax which was present in all mesothelioma cell lines (50). In this study, we have shown that the mesothelioma cell line, M9K, which expresses wild-type p53 (9), is resistant to both asbestos and Fas generated apoptosis whereas the extended lifespan mesothelial cell population and the immortalized, non-tumorigenic MeT-5A cells show selective resistance to asbestos-generated apoptosis (Figure 4C), which suggests that additional changes accumulated during the generation of tumorigenicity in M9K are required for this broad resistance to apoptotic stimuli. Thus, additional changes that may alter the proteolytic cascades required for apoptosis may contribute to the resistance to therapeutic regimens shown by mesothelioma (60,61).

Loss or inactivation of genes whose products contribute to the normal functioning of p53 and pRB are expected to contribute to tumorigenicity. Mutation and loss of p53 have been associated with immortalization of human fibroblasts (62,63). In the present study, normal and extended lifespan cells were stained both for p53 and the downstream p21 whose elevation is associated with senescence (64). Extended lifespan cells did not show altered expression of these genes. Examination of p16INK4a was suggested by the frequent loss of the chromosome 9p region encoding p16INK4a and p14ARF in primary mesothelioma and in mesothelioma cell lines, as well as the association of p16INK4a loss or methylation with extension of lifespan in culture (34,38,65). The data presented here, in agreement with observations on mammary epithelial cells (38,39), show that p16INK4a protein expression is decreased during the rapid growth phase of lifespan extension. However, the gene is neither lost (Figure 9) nor detectably methylated at the 174–185 GC island (Figure 7B). Furthermore, p16INK4a protein is expressed at a high level when cells enter culture crisis (Figure 9B), which indicates that epigenetic mechanisms or processes mediated at the level of transcription control the down regulation.

The current data do not delineate a mechanism for generation of a population exhibiting an extended proliferative lifespan. However, it is clear that p53 can induce growth factor expression (7,66,67) and that human mesothelial cells are responsive to such growth factors (68,69). Indeed, it has been shown that there are great interindividual differences in growth factor responsiveness of normal human mesothelial cells (49). Such differences could be critical in generation of populations exhibiting extended proliferative lifespans and would be expected to protect against apoptosis as well as stimulate proliferation.

In conclusion, we have demonstrated that a subset of normal human mesothelial cells from individual donors can develop an extended proliferative lifespan in response to direct asbestos treatment in vitro, although, for most cells, asbestos treatment leads to cell death through apoptosis or cytotoxicity. Expression of SV40 T antigen is not required for, nor was it shown to, facilitate development of an extended lifespan. The ELP is resistant to asbestos-induced but not Fas-induced apoptosis. This population exhibits decreased p16INK4a protein expression during its proliferative phase. Such a population may represent cells that have already accumulated alterations of significance on the pathway to mesothelioma. The increased breadth of resistance to inducers of apoptosis shown by mesothelioma cell lines indicates that these alterations are specific to asbestos/ mesothelial cell interactions. Future studies of such populations may provide valuable information concerning asbestos-induced changes that are significantly associated with carcinogenesis.

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