Teratogenesis, Carcinogenesis, and Mutagenesis 15:63–71 (1995)

Morphological Transformation Induced by Glass Fibers in BALB/c-3T3 Cells

H.-G. Gao, W.-Z. Whong, W.G. Jones, W.E. Wallace, and T. Ong

Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia

Studies were conducted to determine whether 1) glass fibers can induce morphological transformation in BALB/c-3T3 cells, 2) the transforming activity of glass fibers is related to fiber size, and 3) transformed cells induced by glass fibers possess neoplastic properties. In the transformation assay, BALB/c-3T3 cells were treated with three different types of glass fibers: Manville code 100 (JM-100, Manville Corp., Denver, CO), Owens-Corning AAA-10 (AAA-10, Owens-Corning Corp., Toledo, OH), and Owens-Corning general building insulation (ISL, Owens-Corning Corp.) fibers. The neoplastic properties were investigated using the soft agar cloning and gene transfection methods. All three different glass fibers were cytotoxic at high concentrations and induced dose-related increases in morphological transformation. The transforming activity was inversely related to fiber size, with AAA-10 showing higher activity than JM-100 and JM-100 showing higher activity than ISL fiber. Transformed cells induced by glass fibers exerted anchorage-independent growth (90%) and DNA transfection-mediated transformation (100%). These results indicate that glass fibers are capable of transforming mammalian (BALB/c-3T3) cells in vitro as a function of their physical properties and that glass fiber-induced transformed cells possess preneoplastic characteristics. © 1995 Wiley-Liss, Inc.*

Key words: glass fiber, transformation, BALB/c-3T3, transfection, anchorage independence

INTRODUCTION

Glass fibers, produced as either glass wool or glass filaments, are among the most common man-made fibers. Glass wool fibers are relatively shorter and thinner than glass filaments. Due to the wide range of their applications, such as thermal and acoustical insulation in construction, reinforcement in plastics, light and image transmissions in communication, and popular use in decoration industries [1,2], glass fibers are manufactured in increasing amounts. Consequently, an increase in exposure

Address reprint requests and correspondence to Wen-Zong Whong, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505-2888.

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of workers as well as the general population to glass fibers seems likely. Therefore, it is important to know whether these widely used substances pose a genotoxic hazard to exposed subjects.

It has been reported that glass fibers do not induce gene mutations in *Escherichia coli* and *Salmonella typhimurium* [3], or sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells and human fibroblasts in vitro [4]. However, glass fibers have been shown to induce chromosomal breaks and rearrangements in CHO cells, and chromosomal breaks, fragments, and micronuclei in Chinese hamster lung (V79) cells [5,6]. They also induce a concentration-dependent increase in morphological transformation in cultured Syrian hamster embryo cells [7,8]. Although negative results were found in an inhalation exposure study, a significant increase in cancer induction was observed in rats and hamsters following repeated instillation of glass fibers [2]. Injection of glass fibers into the peritoneal cavity was found to cause sarcomas or mesotheliomas in rats, but not in hamsters [2]. Results of epidemiological studies are also inconsistent. Furthermore, studies on workers in the man-made vitreous fiber (MMVF) industry have failed to demonstrate a correlation between fiber exposure and an incidence of lung cancer or non-malignant respiratory diseases [9]. However, an increase in cancer incidence has been reported in workers in the fiber production industries of Italy [10]. Also, in an international investigation at 13 European MMVF-producing plants, an elevated incidence of lung cancer was found in workers with a 30- or more year employment history [11].

It is conceivable that the evidence detailing the carcinogenic activity of glass fibers is still inadequate. Thus, further studies regarding their carcinogenic potential need to be conducted. In the current study, the cell transforming activity of these man-made fibers was investigated using BALB/c-3T3 cells and the neoplastic properties of their transformed cells were also characterized.

MATERIALS AND METHODS

Fiber Samples

Three types of glass fibers—Owens-Corning AAA-10 microfiber (AAA-10, Owens-Corning Corp., Toledo, OH), Manville code 100 (JM-100, Manville Corp., Denver, CO), and Owens-Corning general building insulation (ISL, Owens-Corning Corp.)—were used in this study. Bulk samples of glass fibers were reduced in length for use in the cell transformation experiments by using a knife mill. The length and width distributions were obtained by light microscopy for the building insulation sample and by transmission electron microscopy for the two types of microfibers (Fig. 1).

Media and Reagents

Eagle's minimum essential medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). The eukaryotic transfection kit and Nobel agar were purchased from Stratagene Co. (La Jolla, CA) and Difco Laboratories (Detroit, MI), respectively. Phosphate buffered saline (PBS) was purchased from Microbiology Systems (Cockeysville, MD) and proteinase K and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO).

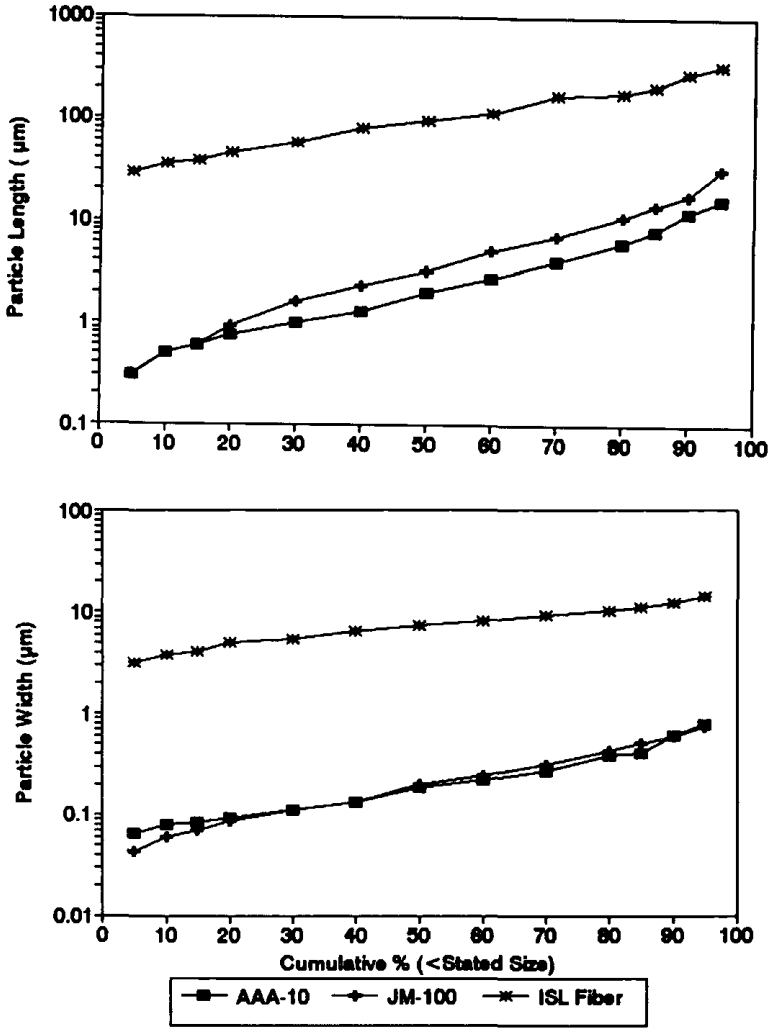

Fig. 1. Particle size distribution of three different glass fibers: JM-100 (+), AAA-10 (■), and ISL (*). Results were derived from two independent experiments.

Cell Culture

BALB/c-3T3 cells provided by Dr. E.J. Matthews (Hazleton Laboratories, Kensington, MD) were used for morphological transformation assays, whereas NIH-3T3 cells purchased from the American Type Culture Collection (Rockville, MD) were used for transfection studies. Stock cells were maintained in liquid nitrogen. BALB/c-3T3 cultures were initiated by rapid thawing of a stock vial containing $\geq 1 \times 10^6$ cells at 37°C and grown in MEM and DMEM, respectively, supplemented with 1% L-glutamine, 1% penicillin/streptomycin, 50 mM gentamicin, and 10% FBS in 5% CO_2 at 37°C. Cells in cultures were routinely passaged twice a week with a split ratio of 1:6. Cell cultures used for experiments were passaged at least 3, but no more than 12 times.

Cell Transformation Assay

The cell transformation assay was carried out as described by Matthews [12]. Concentrations of glass fibers for the cell transformation assay were selected based

on data obtained from a preliminary cytotoxicity assay. Five concentrations that gave approximately 10–80% survivors compared to the solvent control were performed for each type of glass fiber. Solvent controls were culture medium and dimethylsulfoxide (DMSO), whereas BaP_1(0.2 μg/25 cm^2 flask) was used as the positive control. For the experiment, a series of 25 cm^2 flasks were seeded with 2.5×10^4 BALB/c-3T3 cells/flask and incubated in 5% CO_2 at 37°C in a humidified atmosphere. Each treatment set consisted of at least 20 culture flasks. A clonal assay was conducted simultaneously with the transformation assay to verify the cytotoxicity of glass fibers. After 24 h of growth, the seeded cell cultures were treated with each of the three different glass fibers and incubated for 72 h. Upon completing the exposure period, cells were washed 2 times with PBS, refed with fresh culture medium, and then incubated for 4–5 weeks with medium changes twice per week. At the end of the incubation, cells from transformed foci were isolated and cultured (for transfection and anchorage-independent growth studies) and the flasks were then stained for scoring of the type III transformed foci.

Anchorage-Independent Assay

The soft agar suspension medium was prepared as described previously [13,14]. Briefly, 3 g of Noble agar (Difco) in 500 ml of plain MEM (no supplements) was autoclaved for 20 min. After cooling to 50°C, 10% FBS, 1% L-glutamine, and 1% antibiotics were added to the agar solution. Fifteen milliliters of the agar/medium solution was pipetted into each 100 mm Petri dish and set aside to solidify. For the top agar, 3% sterile molten agar solution was prepared with MEM. In 30 ml of complete medium, 10^4 parent or glass fiber-transformed cells and 3 ml of molten agar solution were added, and then 5 ml of cell/agar mixture was pipetted onto each of 6 bottom agar plates and incubated for 4 weeks with no medium change. At the end of the incubation, colonies larger than 50 cells were scored as anchorage-independent clones.

Transfection Assay

The calcium-phosphate precipitation method [15] was employed for the DNA transfection assay with minor modifications. Briefly, a series of 75 cm^2 culture flasks were seeded with 5×10^5 NIH-3T3 cells/flask in 10 ml supplemented DMEM. After an overnight incubation, 30 μg of DNA extracted from transformants was diluted with distilled, deionized water to 450 μl. Fifty microliters of solution 1 (2.5 M $CaCl_2$) and 50 μl of solution 2 (2× N,N-bis[2-hydroxyethyl]-2-amino-ethane sulfonic acid and buffered saline) were added to DNA, gently mixed, and allowed to incubate for 10–20 min at room temperature for the precipitation of DNA. After incubation, the DNA precipitate was mixed carefully and then added drop by drop to the preseeded NIH-3T3 cell culture. The plate was gently swirled to evenly distribute the precipitate. Cell cultures were incubated for 12–24 h at 37°C in a 3% CO_2 incubator. At the end of incubation, medium was removed and cultures were rinsed 2 times with PBS. Fresh culture medium was added and cells were further incubated for 24 h. Cells in each culture were then split at a 1:20 ratio and incubated for 4 more weeks with medium changed biweekly. At the end of incubation, transformed type III foci in the culture were scored as DNA transfectants.

Statistical Analysis

The cell transforming activity was analyzed according to the method of Matthews [12], in which the data for the number of transformed foci in each culture flask were

$\log_{10}$-transformed and the levels of significance were determined using the t-test. For both the anchorage-independent growth and gene transfection assays, a one-tailed Student's t-test was used for the analysis.

RESULTS

The cytotoxicity of glass fibers was measured by the relative colony-forming efficiency (RCE), which was defined as the percentage of the control colony-forming efficiency (CFE) present in the treated cells (RCE = control CFE/treated CFE × 100%). As shown in Figure 2A, all three types of glass fibers were cytotoxic to BALB/c-3T3 cells. At the higher concentrations (>10 μg/cm^2), both JM-100 and AAA-10 appeared to be more cytotoxic than ISL fiber. In the cell transformation assay, all

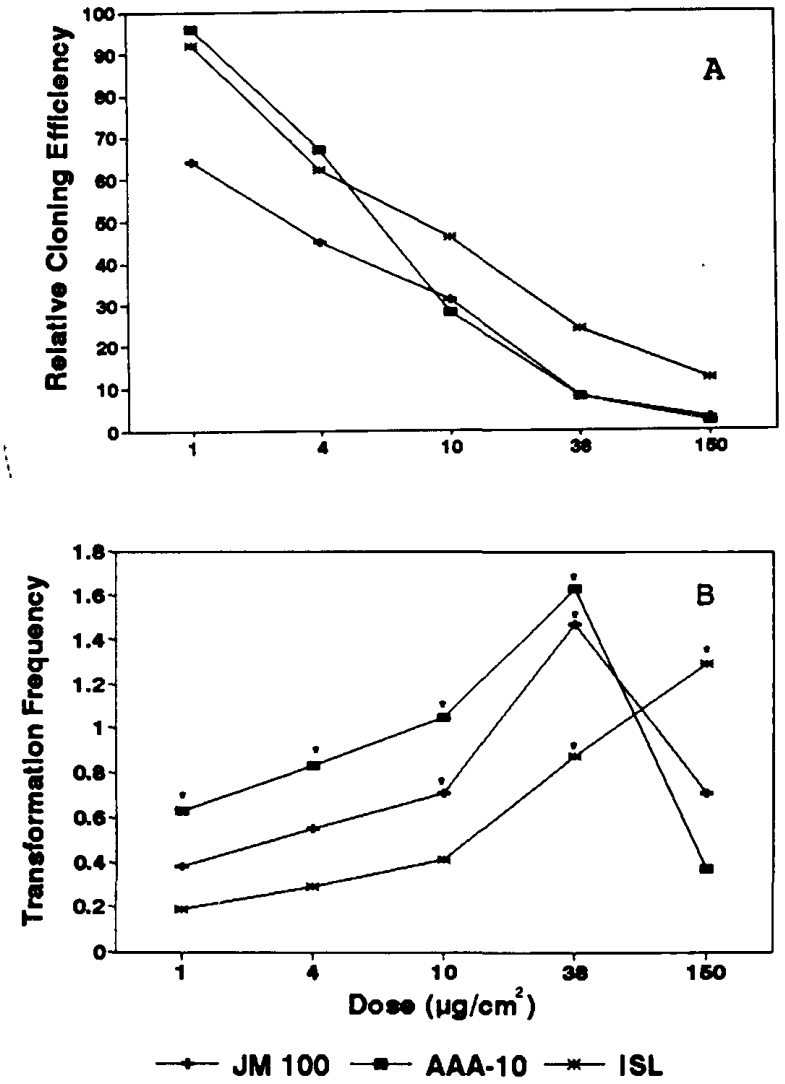

Fig. 2. Cytotoxic (A) and transforming activities (B) of glass fibers JM-100, AAA-10, and ISL in BALB/c-3T3 cells (*significant). Transforming activities of solvent control (MEM) and positive control (BaP 0.2 μg/flask) were 0.11 and 2.64 foci/flask, respectively. The experiment was repeated three times independently.

three types of transformed foci were found in which type III foci, consisting of dense, round cells and criss-cross cells at the periphery, were considered to be representative of true transformation. A concentration-related transformation was observed in BALB/c-3T3 cells treated with three different glass fibers (Fig. 2B). The concentrations which caused a significant increase in cell transformation were found from 10 $\mu g/cm^2$ for AAA-10, 10 $\mu g/cm^2$ for JM-100 and 38 $\mu g/cm^2$ for ISL fibers. The transformation potencies among the three types of fibers were in the order of AAA-10 > JM-100 > ISL fibers.

The presence of anchorage-independent growth in glass fiber-induced transformed cells was determined by colony-forming efficiency in soft agar medium. In this medium, only cells carrying anchorage-independent growth divided and formed visible colonies. Table I shows CFE of glass fiber-transformed BALB/c-3T3 cells in soft agar. The transformed cells induced by glass fibers demonstrated a greatly enhanced growth ability in soft agar compared to non-transformed BALB/c-3T3 cells. The average CFE of the glass fiber-transformed cells in soft agar ranged from 5.04 to 9.74%, which was significantly higher than the 0.34% of non-transformed cells.

In the transfection assay, DNAs from 10 foci transformed by AAA-10 were analyzed using the NIH-3T3 host cells. Results demonstrate that all 10 foci significantly induced morphological transformation in the host cells (Table II). The frequency of transfection-induced transformation was 0.23–2.2 foci/μg DNA of glass fiber-transformed cells, 2–22 times higher than that of negative control (normal BALB/c-3T3 cells). The transfection-mediated transformation by DNA from glass fiber-transformed cells was morphologically indistinguishable from that generated by transfection of plasmid DNA from T-24 human bladder carcinoma (positive control).

TABLE I. Growth of Glass Fiber-Induced Transformed Cells in Soft Agar Medium

	1,000 cells	
Cells[a]	Colonies (mean ± SD)	Cloning efficiency* (%)
J1 (JM-100)	82.4 ± 4.6	8.24
J2	87.3 ± 4.4	8.73
J3	85.6 ± 3.7	8.56
J4	78.4 ± 2.5	7.84
J5	79.4 ± 3.6	7.94
A1 (AAA-10)	55.4 ± 7.3	5.54
A2	89.5 ± 6.8	8.95
A3	97.4 ± 5.6	9.74
A4	92.1 ± 6.7	9.21
A5	84.6 ± 4.6	8.46
I1 (ISL fiber)	50.4 ± 5.8	5.04
I2	80.5 ± 3.7	8.05
I3	72.6 ± 4.6	7.26
I4	65.3 ± 3.5	6.53
I5	56.4 ± 4.6	5.64
BALB/c-3T3	3.4 ± 2.7	0.34

[a]Fifth passage.

*All values for transformed cells are significantly higher than non-transformed BALB/c-3T3 cells ($P < 0.05$).

TABLE II. Transfection Efficiency of DNA From Transformed BALB/c-3T3 Cells Induced by AAA-10 Glass Fibers

Sample No.	Foci /flasks[a]	Transformation frequency[b] (mean ± SE)
1	22/20	0.73 ± 0.16**
2	11/20	0.37 ± 0.10*
3	7/20	0.23 ± 0.10**
4	25/20	0.83 ± 0.19**
5	42/20	1.40 ± 0.23**
6	66/20	2.20 ± 0.18**
7	21/20	0.70 ± 0.16**
8	11/20	0.37 ± 0.08**
9	28/20	0.93 ± 0.15**
10	13/20	0.43 ± 0.19*
BALB/c-3T3[c]	3/20	0.10 ± 0.06
H-*ras* oncogene[d]	16/20	16.00 ± 4.94**

[a]Number of type III foci.
[b]Calculated according to Matthews [12].
[c]Non-transformed cell control.
[d]Positive control (T-24).
*$P < 0.05$.
**$P < 0.01$.

DISCUSSION

Results from these studies clearly demonstrate that glass fibers induced transformation in a concentration-dependent manner. However, there were differences in transforming activity among the three different glass fibers studied, in which the sample of the shortest microfibers (AAA-10) showed the highest transforming potency, whereas the sample of the thick and longest length (ISL fiber) showed the lowest activity. This seems to indicate that glass fiber-induced transformation may be related to fiber size. These observations are comparable to previous findings using other transformation systems, which showed that both thin (code 100) and thick (code 110) glass fibers induced cell transformation in cultured Syrian hamster embryo cells and that the cell-transforming activity was higher with thin than with thick glass fibers [7,8]. Stanton et al. [16,17] also demonstrated a dimensional dependence of fiber carcinogenesis in which the inactivation of short and large fibers by phagocytes (e.g., macrophages) was suggested to account for the non-correlation between the number of fibers and tumor probability for fibers with width larger than 1.5 μm and length smaller than 4 μm. Since the in vitro transformation assay system was used in our studies, phagocytic inactivation would not occur. The finding of transforming activity of ISL fiber with a greater width and length may be attributed to the presence of a small fraction of small size fibers in the sample.

All transformed foci induced by three different glass fibers showed typical characteristics of morphological transformation, such as random cellular orientation, cell piling up, and high saturation density [12,18]. The transfection study revealed that DNAs from glass fiber (AAA-10)-transformed cells induced transfection-mediated transformation in host NIH-3T3 cells, suggesting that the cells transformed by glass fibers contain transforming genes. Furthermore, soft agar cloning analysis provided evidence that glass fiber-induced transformed cells possessed another transformed

property, anchorage-independent growth [19]. It has been reported that the ability to grow in soft agar (anchorage-independent growth) is well correlated with tumorigenicity in vivo [19–24]. Our study, therefore, indicated that transformed cells induced by glass fibers carried characteristics of preneoplastic transformation.

Carcinogenesis has been proposed to be a multistep process [25] and it may involve the activation of oncogenes and/or the inactivation of tumor suppressor genes [26]. The actual mechanism of cell transformation induced by glass fibers is unknown. With the Syrian hamster embryo cell system, Oshimura et al. [8] showed that glass fibers were positive in inducing both morphological transformation and chromosomal changes. Recent studies by Liu et al. [6] showed that glass fibers are capable of inducing micronuclei in V79 cells. Using kinetochore analysis, this group further demonstrated that glass fiber-induced micronuclei resulted from spindle fiber damage. Based on these findings, it can be postulated that cell transformations induced by glass fibers may be related to chromosomal abnormalities [7,8]. It has also been reported that transfection-mediated transformation often results from the transference of activated proto-oncogenes (e.g., *ras*) present in donor DNAs to their respective host cells [27,28]. Since positive results were found for gene transfection with DNAs from glass fiber-transformed cells, transforming genes derived from the alteration of proto-oncogenes and/or tumor suppressor genes may also contribute to cell transformation induced by glass fibers. To provide insight into the mechanisms of potential glass fiber carcinogenesis, molecular analyses of these genes in glass fiber-induced transformed cells are now in progress.

ACKNOWLEDGMENTS

The authors thank Drs. Ann Hubbs and Craig Stamm for their critical review and Mrs. Helen Michael for her secretarial assistance.

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