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TRANSLOCATION OF CHROMOSOMES 16 AND 18 IN OXYGEN RADICAL-TRANSFORMED HUMAN LUNG FIBROBLASTS

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Human lung fibroblasts (MRC5) were treated chronically with the oxygen radical-generating system hypoxanthine plus xanthine oxidase and assayed for malignant transformation in the soft agar colony assay. After a thrice weekly exposure to oxygen radicals for 4 and 5 weeks, there was a significant number of transformants compared to controls. In 4 separate experiments, karyotypes of the malignant transformants were examined. 22/75 metaphases exhibited karyotypic abnormalities and in 13/22 of the abnormal karyotypes, a t16:18 (p13.3,q21) translocation was observed. This genetic lesion may represent a marker for oxygen radical-induced malignant transformation in mammalian cells.

Oxygen-derived free radicals may be involved in both the initiation and promotional stages of multistage carcinogenesis. Phagocyte-generated oxygen radicals have been shown to induce malignant transformation of C3H10T1/2 cells in an *in vivo* malignant transformation assay (1) and enzymatically-generated radicals promote the transformation of mouse embryo C3H10T1/2 clone 8 cells *in vitro* (2). Although oxygen radicals induce a variety of genetic lesions (sister chromatid exchanges, DNA strand breaks and base modifications, mutations)(3-5) in cultured cells, a causative role for oxygen radicals in the transformation process remains to be defined.

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In this study, human lung fibroblasts were chronically exposed to an oxygen radical-generating system and assayed for malignant transformation. Karyotypes of the transformed cells were prepared and examined for a non-random genetic marker for oxygen radical-induced malignant transformation.

METHODS

Cell Cultures. MRC5 fetal human lung fibroblasts are obtained from the American Type Culture Collection, Rockville, MD. Cells are received at passage 18 and are not used beyond passage 24. 10^5 cells are incubated in T75 flasks at 37°C in MEM medium supplemented with fetal bovine serum (10% v/v), penicillin/streptomycin (5% v/v) and HEPES buffer (5% v/v). Doubling time is 26 hrs and confluence is reached in approximately one week (10^7 cells). Cells (3 T75 flasks/data point) are treated 3 times/wk for 10 min with hypoxanthine (7 μg/ml) and xanthine oxidase (15 μg/ml). At the completion of each incubation, the supernatant is washed out and replaced with medium. Treatment continues for 5 wks and the cells are passaged at one wk intervals upon reaching confluence. At each passage, 2 T75 flasks per data point containing 1-2 x 10^5 cells are established. At 4 and 5 wks of treatment, cells are passaged, grown to confluence and passaged a 6th time, distributed in soft agar and observed up to 3 weeks for the development of anchorage independent colonies.

Soft Agar Colony Assay (6). Six-well plastic dishes (well area 9 cm^2) are used. The 0.5 agar medium is prepared by mixing 80 ml of agar solution (1.25% Difco Bacto Agar), 20 ml fetal calf serum, 20 ml tryptase phosphate broth and 80 ml double-strength (2X) MEM and kept at 44°C. 2 ml of this 0.5% agar medium is pipetted into each well and allowed to solidify as a basal layer. The 0.3% soft agar medium containing the treated and control MRC5 cells is prepared by mixing 1 volume of each cell suspension in medium at 37°C with 2 volumes of agar medium at 44°C. Mixing is achieved by gentle swirling and 1 ml is overlaid on the basal layer. Approximately 10^4 cells are plated per well (2 dishes per data point). Colony formation (0.1mm-0.2mm) is observed and counted under a microscope without staining. For photography, the samples are incubated with 2-p-iodophenyl-3-p-nitro-phenyl-5-phenyl tetrazolium chloride (1mg/ml in distilled water) for 20 hours (7).

Karyotypes (Giemsa-Trypsin Banding)(8). Confluent T75 flasks after 4 and 5 weeks of treatment are trypsinized and distributed in fresh flasks (10^6 cells/flask) in 10 ml RPMI/MEM medium supplemented with 10% fetal bovine serum, GCT conditioned medium, penicillin/streptomycin and L-glutamine. After an overnight incubation, the cells are treated with colcemid for 20 min and then heated with hypotonic (0.068M KCl) solution followed by 3 fixative changes. The cells then are trypsinized, centrifuged and the pellet is dropped onto clean glass slides and aged overnight at 60°C. The slides are laid flat in a dish and flooded with a 15% H_2O_2 solution for 7 min. The slides are rinsed thoroughly with distilled water and dried on a hot plate (50°C.) for 1 hr. The slides then are treated with working trypsin solution in a Coplin jar for 3-30 secs. Slides are rinsed in Hank's Balanced Salt Solution with constant agitation and then rinsed in distilled water. They are stained with 4% Giemsa, rinsed and air-dried.

RESULTS

As shown in Figure 1, oxygen radical-induced malignant transformation of MRC5 cells occurred in a dose-dependent fashion as evidenced by the

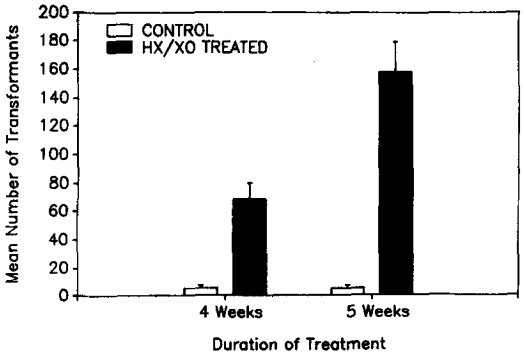

Figure 1. Effect of oxygen radicals on transformation of MRC5 cells. Controls consisted of MRC5 cells alone with and without hypoxanthine or xanthine oxidase alone.

increased number of transformants at 4 and 5 weeks ($p<.0005$ vs. control). Each data point represents the mean of four experiments. Counting was performed both before and after staining (see methods) and 6 wells were counted per data point.

Table 1 illustrates the karyotypic abnormalities observed and their frequency. The t16:18 (p13.3,q21) abnormality was a non-random event in

Table 1. Karyotypes of MRC5 cells chronically treated with oxygen radicals

Control Group (#)	Treated Group (#)
46, XY (67)	46, XY (53)
46, XY inv 3 (1)	46, XY t16:18 (p13.3,q21) (13)
46, XY 11p-,+2 mar (1)	46, XY 6p+ (1)
46, X,-Y,+1 mar (1)	46, XY -18,+2 mar (1)
46, XY t2:13,2p- (1)	45, XY -6 (1)
44 XY -8,-17,del8(q24.1) (1)	46, XY del 6(p11) (1)
	46, XY del 13(q14) (2)
	46, XY t9:14(q12,q31.3) (1)
	48, XY -2,-16,+4 mar (1)
	48, XY +double min,+1 mar (1)

Abbreviations: inv - inversion; mar - marker; del - deletion; double min - double minute.

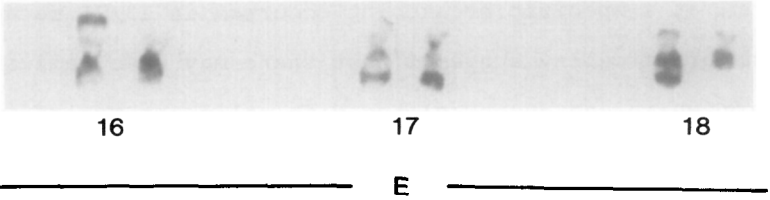

Figure 2. Representative portion of a karyotype illustrating the 16:18 translocation.

that the translocation was observed in metaphases from transformed cells from separate flasks and separate experiments (18-20 metaphases/ experiment). Five abnormal metaphases occurred in the control group (total, 72 metaphases), but none exhibited the 16:18 translocation. A portion of a karyotype illustraing the t16:18 (p13.3,q21) abnormality is shown in Figure 2.

DISCUSSION

Chronic inflammation has been associated with human malignancy and is characterized by the infiltration of tissues with polymorphonuclear leukocytes and macrophages which discharge a variety of oxygen radicals into the area. It is not clear how these short-lived radicals produce damage to nuclear DNA, but extracellularly-generated oxygen radicals clearly have been shown to be genotoxic (3-5).

The role of oxygen radicals in the transformation process is not well-defined. In addition to our work demonstrating the participation of oxidants in the malignant transformation of mammalian cells *in vitro* and *in vivo* (1,9), enzymatically-generated radicals have been shown to promote the transformation of C3H10T1/2 cells *in vitro* (2). Karyotypic analyses of these transformants were not performed, but in irradiation-transformed Golden hamster embryo cells, trisomy of chromosome 7 was observed in all of the anchorage independent clones (10).

In this study, we were able to transform a human lung fibroblast cell line with chronic exposures of enzymatically-generated oxygen radicals. Studies to determine the radicals most important in this process are underway. However, the observation that a specific chromosomal

translocation, at a specific breakpoint occurred in 13/75 metaphases from radical-transformed cells, suggests that there may be a radical-specific genetic lesion which can be correlated with malignant transformation in future experiemnts. Since the cells were harvested for preparation of the karyotypes prior to establishing them in soft agar, not every metaphase examined was from a transformed clone. Once a cell line is established from the radical-induced transformants, karyotypes from specific transformed clones will be examined.

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