Mutation Research, 262 (1991) 51-55
Elsevier

MUTLET 446

Characterization of the in vitro unscheduled DNA synthesis assay in primary lung cells of the rat

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(Received 5 June 1990)
(Revision received 24 August 1990)
(Accepted 27 August 1990)

Keywords: DNA repair; Primary lung cells; Alveolar macrophages; (Rat); Unscheduled DNA synthesis (UDS)

Summary

The in vitro unscheduled DNA synthesis (UDS) assay has been evaluated in rat primary lung cells with known genotoxicants. The autoradiographic method was employed to detect UDS in both alveolar macrophages and primary pulmonary cells. Data of a time course study revealed that a high radioactive labeling of DNA repair was achieved after a 16-h incubation with [^{3}H]thymidine. Coupled with low serum (1%), hydroxyurea at the concentration of 20 mM inhibited regular DNA synthesis in primary lung cells in a satisfactory manner (81–88% inhibition). With this protocol, a dose-related increase in UDS was induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 2-aminoanthracene in both rat alveolar macrophages and primary lung cells. The results suggest that primary rat lung cells in culture possess DNA-repair ability and that the UDS assay may be useful for assessing the pulmonary genotoxic effect of chemicals in this cell system.

It is well known that genotoxic agents can be present in the environment as gases, vapors and airborne particles. Hence, inhalation becomes an important route for exposure to such toxic substances and the lung can be one of the major targets for their genotoxic effects. Therefore primary lung cells, which may retain most of their original metabolic ability (Devereux and Fouts, 1981; Li et al., 1983; Shimizu et al., 1984; Aune et al., 1985; Deilhaug et al., 1985), may provide a useful cell system for evaluating the pulmonary genotoxicity of chemicals.

Evidence for DNA repair resulting from DNA damage has been documented (Cleaver and Painter, 1968). DNA repair, which is nonsemiconservative in nature, has been called unscheduled DNA synthesis (UDS) (Djordevic and Tolmach, 1967). Since genotoxic agents may generate damages in DNA, measurement of UDS provides a

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useful assay system for detecting and assessing such hazardous substances. To date, the UDS assay has been used for the evaluation of genotoxicity of chemicals in a variety of cell types (Sega, 1979; Mitchell et al., 1983; Doolittle and Butterworth, 1984; Doolittle et al., 1984), especially in liver cells (Williams et al., 1989). However, the usefulness of this assay in primary lung cells (Cheng et al., 1983; Haugen et al., 1986) has not been adequately explored.

Recently, we have been engaged in the development of a multiple genetic endpoint genotoxicity assay using rat primary lung cells (Whong et al., 1990). In this report, the UDS assay with autoradiographic technique in primary lung cells was explored. The sensitivity of the assay in vitro was evaluated with known genotoxicants in both alveolar macrophages and primary pulmonary cells of rats.

Materials and methods

Animals. Male CD rats of 6–8 weeks old (Charles River Breeding Lab., Inc., Wilmington, MA) were used for the isolation of alveolar macrophages and lung cells.

Chemicals. Protease (Type 14) was obtained from Sigma Chemical Co. (St. Louis, MO), whereas 2-aminoanthracene (2AA) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were from Aldrich Chemical Co. (Milwaukee, WI). Medium F12 and Hanks' balanced salt solution (HBSS) were purchased from Gibco Laboratories (Grand Island, NY) and [^{3}H]thymidine was obtained from ICN Biomedicals, Inc. (Irvine, CA).

Preparation of primary lung cells. After rats were anaesthetized, alveolar macrophages were harvested by bronchoalveolar lavage with Ca^{2+}- and Mg^{2+}-free HBSS (6–10 ml/lavage and 5 lavages/rat) (Jones et al., 1982). Lungs were then perfused with saline to remove the blood via pulmonary artery. 8- and 2-ml volumes of protease (2 mg/ml) were injected into each lung through trachea and pulmonary artery, respectively. The lungs were incubated at 4°C for 16 h, then cut individually into small pieces (1–2 mm^2), and incubated at 37°C for 30 more min. At the end of incubation, 4 ml F12 medium and 1 ml each of fetal bovine serum (FBS) and DNAase (0.6 mg/ml) solutions were added to each digested cell suspension and the cell suspensions were filtered with nylon blotting and silk cloths (100 and 305 meshes, respectively). The separated lung cells and alveolar macrophages were cultured in 25-mm^2 flasks and 60-mm dishes, respectively, each containing 5 ml F12 medium supplemented with 10% FBS, 100 U penicillin/ml, 100 μg streptomycin/ml, and 0.25 μg fungizone/ml.

UDS assay. The autoradiographic method was employed to measure UDS (Williams, 1977; Williams et al., 1989). After 4-day culturing in F12 medium containing 10% FBS and 1-day culturing in the same medium of 1% FBS, primary lung cells were treated with designated concentrations of MNNG and 2AA in the presence of 10 mCi [^{3}H]thymidine/ml at 37°C for 16 h. Hydroxyurea (20 mM) was also added 1 h before chemical treatments for inhibiting DNA replication. The same conditions were also applied for controls, except that solvent (DMSO) instead of genotoxicants was added. At the end of treatment, test compounds and [^{3}H]thymidine were removed and cells were incubated for 2 h in F12 medium which was supplemented with cold thymidine (25 μg/ml). The cells were then harvested and subjected to hypotonic treatment (0.075 M KCl) at 37°C for 8 min. Cells smeared on slides were fixed with methanol for 10 min and coated with photographic emulsion (NTB-2), which was diluted with H_2O (1:1). After exposure in the dark at 4°C for 2 weeks, autoradiograms were developed and slides were stained with Giemsa (0.04%). Net grains per nucleus were obtained by subtracting cytoplasmic grains from nuclear grains. The number of cytoplasmic grains were determined by counting 2 nuclear-sized areas in the cytoplasm of each cell. 100 cells per treatment were scored and nuclear net grains equal to or more than 5 were considered to be positive response (Mirsalis and Butterworth,

1982; Working et al., 1986). Percent cells in repair were also measured as the percentage of cells with net nuclear grains $\geqslant 5$.

For alveolar macrophages, treatment was conducted after one-day culturing. Treatment conditions were the same as those described above for primary lung cells, except that no hydroxyurea was used.

Results and discussion

With the lavage method used, approx. $1\text{-}3 \times 10^6$ alveolar macrophages/rat were isolated, in which at least 92% of macrophages were viable as determined by trypan blue dye-exclusion. In regard to the separation of lung cells, cold digestion of the lung with protease gave an excellent cell dissociation, resulting in a high yield of separated viable lung cells ($3.4\text{-}4.8 \times 10^7$ cells/lung of 82–91% viability). The efficiency of cell dissociation with this method appears to be comparable to the combined enzyme (trypsin and collagenase) digestion method used previously (Whong et al., 1990). The cold enzyme digestion method has been used for isolation of the airway epithelial cells from rabbit tracheas (Wu and Smith, 1982).

After 4-day culturing, a considerable number of lung cells were in replication. In order to inhibit the semiconservative DNA synthesis, the effectiveness of low serum and DNA-synthesis inhibitor hydroxyurea at different concentrations was examined. Hydroxyurea alone, at the concentration as high as 20 mM did not sufficiently inhibit regular DNA replication in the primary lung cells (Table 1). However, in conjunction with a low concentration (1%) of serum, satisfactory inhibition was obtained by the same concentration of hydroxyurea. A low serum content (0.5%) coupled with hydroxyurea (10 mM) has been used for blocking cultured human cells into S phase (Mitchell et al., 1983). Since hydroxyurea at the concentration of 20 mM does not inhibit unscheduled DNA synthesis (Mirsalis and Butterworth, 1982), this concentration was used for the experiments with primary lung cells. As to alveolar macrophages, no inhibition of DNA replication was necessary, because only a few

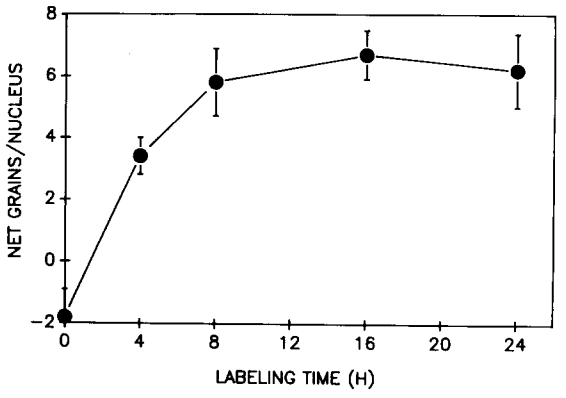

Fig. 1. Induction of unscheduled DNA synthesis by MNNG (5 mg/ml) as a function of [^{3}H]thymidine labeling time (bar = standard error).

macrophages entered S phase (0.8–7%) during a 16-h ^{3}H labeling.

Fig. 1 shows the effect of radioactive labeling time on the detection of UDS in primary lung cells. MNNG-induced UDS was detected as early as 4-h labeling. The UDS increased as labeling time increased and then leveled off after 16 h. This result suggests that a labeling time of 16 h is essential to obtain a maximal induced DNA repair in the UDS assay system. Because DNA-damaging substances could induce long-lived DNA lesions in mammalian cells, it might require a longer time to remove these DNA damages (Singer, 1979; Door et al., 1985). Lengthening the labeling time to increase sensitivity of UDS detection in liver primary cell cultures has been reported by a number of in-

TABLE 1

EFFECT OF HYDROXYUREA AND SERUM ON THE INHIBITION OF SCHEDULED DNA SYNTHESIS[a]

Hydroxyurea (mM)	% cells in S phase	
	10% serum	1% serum
0	82–88	75–81
10	54–73	40–52
20	38–47	12–19

[a] 4-day-old cultures of primary rat lung cells were labeled with [^{3}H]thymidine for 16 h.

TABLE 2

UNSCHEDULED DNA SYNTHESIS IN RAT PRIMARY LUNG CELLS TREATED WITH MNNG AND 2AA[a]

Genotoxicant	Concentration (μg/ml)	Net grains/ nucleus (Mean ± SE)	% Cells with net nuclear grain ≥5 (Mean)
MNNG	0	−3.1 ± 0.3	3.7
		8.5 ± 0.7[b]	79.4
	10	17.1 ± 0.9[b]	96.7
	20	28.0 ± 1.1[b]	99.5
2AA	0	−1.3 ± 0.1	5.6
	2.5	4.3 ± 0.5	38.9
	5	13.5 ± 1.3[b]	83.4
	10	17.9 ± 1.5[b]	90.8

[a] 4-day-old cultures were treated with test agents for 16 h in the presence of [^{3}H]thymidine, hydroxyurea (20 mM) and 1% serum.
[b] Positive response (net grains/nucleus ≥5).

vestigators (Williams, 1977; Yager and Miller, 1978; Bargknecht et al., 1988).

With a 16-h exposure/labeling time, UDS induced by 2 known genotoxicants MNNG and 2AA, was investigated in rat alveolar macrophages and primary lung cells. The UDS measurements in the present study were relatively similar to those used for the hepatocyte/DNA repair (Williams, et al., 1989). As shown in Table 2, a dose-related increase in UDS following exposure to MNNG, a direct-acting genotoxicant, and 2AA, a progenotoxicant, without exogenous activation was demonstrated in primary lung cells. Activation of progenotoxicants by primary lung cells of the rat or other experimental animals has also been demonstrated in other genetic endpoints (Shimizu et al., 1984; Aune et al., 1985; Whong et al., 1990). Comparable results were also observed for these genotoxic agents in alveolar macrophages (Table 3). However, the positive response to 2AA by alveolar macrophages was obtained only when cells were treated with the agent in the presence of S9 activation. As for the percentage of cells performing DNA repair, both cell systems displayed a high increase in repairing cells induced by 2AA and MNNG, indicating that rat-lung cells in culture are capable of repairing DNA damages. In vivo DNA repair in rat lung cells has also been reported previously (Cox and Irving, 1975; Haski and Stewart, 1979). Results from the present study suggest that these lung cells may also be suitable for the UDS assay system to study the pulmonary genotoxicity of environmental and industrial agents.

TABLE 3

UNSCHEDULED DNA SYNTHESIS IN RAT ALVEOLAR MACROPHAGES TREATED WITH MNNG AND 2AA[a]

Genotoxicant	Concentration (μg/ml)	Net grains/ nucleus (Mean ± SE)	% Cells with net nuclear grain ≥5 (Mean)
MNNG	0	−2.2 ± 0.6	6.0
	5	10.0 ± 0.8[b]	84.0
	10	15.2 ± 1.1[b]	90.2
	20	25.6 ± 1.0[b]	98.4
2AA[c]	0	−3.0 ± 0.4	2.0
	2.5	3.1 ± 0.5	38.9
	5	5.8 ± 0.8[b]	68.5
	10	9.0 ± 0.7[b]	85.2

[a] 1-day-old cultures were treated with test agents for 16 h without a DNA-synthesis inhibitor (hydroxyurea).
[b] Positive response (net grains/nucleus ≥5).
[c] Treated with S9 activation (5% Aroclor-1254-induced rat-liver homogenate) for 4 h.

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Communicated by J.M. Gentile