NEW BIOMEDICAL TECHNOLOGIES

Use of Cultured Human Fibroblasts for Rapid Evaluation of Cytotoxic Effects of Bioactive Substances

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A test system for detecting cytotoxic effects of bioactive substances based on human fibroblast culture is proposed. The effects of acrylamide, streptomycin, cycloheximide, sodium dodecyl sulfate, sanguiritrine, and ethanol were evaluated by organic stain binding. Typical dose-effect relationships were detected for all substances except cycloheximide. The proposed test system can be used for screening of bioactive substances in preclinical trials.

Key Words: *human fibroblasts; test system; cytotoxicity*

The need for a standard protocol of preclinical and clinical trials of bioactive substances (BAS) proposed for the use as drugs or food additives (foodstuffs) is obvious [1,4,5,7]. Mammalian and particularly human cell cultures can play an important role in preclinical studies. Such models are now used for evaluating drug effects on metabolic processes and cell growth, for detecting side effects, and evaluating optimal doses for humans [6,8-12]. We investigated the possibility of evaluating the cytotoxic effects of BAS in a test system based on cultured postnatal diploid human fibroblasts.

MATERIALS AND METHODS

Cultures of postnatal diploid human fibroblasts IMG 1540 and IMG 1398, prepared as described previously [2], were used for creating the test system.

The cells were cultured in DMEM (PanEco) supplemented with 5% cattle serum and 5% human cord blood serum (PanEco) in Carrel's glass flasks, plastic disposable flasks (Costar), or in 96-well plates (Nunc). Methylene blue vital stain [3] and Giemsa stain [4] (Merck) were used.

The following BAS were analyzed: streptomycin, cycloheximide (Merck), acrylamide (LKB), sanguiritrine, sodium dodecyl sulfate (Serva), and ethanol. Sagviritrine preparation containing sanguiritrine and chelerythrine in the form of sulfomethylates with quaternary nitrogen atom was obtained at the Center of Biomedical Technologies. Other Russian-made reagents were of chemically pure and extrapure grades.

For detecting the relationship between cell number per well and the intensity of methylene blue staining, the cells seeded at different density in 96-well plates were incubated for 24 h at 37°C, vitally stained with methylene blue (25 μl per each well) for 1 h, washed with water, and dried on air. Optical density was measured at 594 nm on an EFOS 9305 electrophotometer.

For evaluating the effects of BAS on proliferative activity of human fibroblasts, the agents were added to cells growing in 96-well plates, and after 4 days the cultures were stained with methylene blue as described above. The morphology of growing cells was examined under an MBI-3 microscope (LOMO) adapted as inverted microscope with a special attachment.

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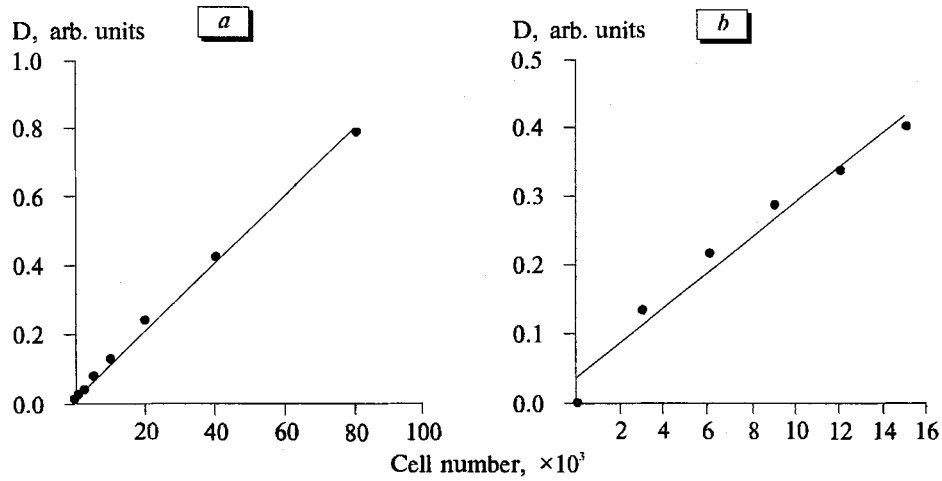

Fig. 1. Optical density (D), arithmetic mean for 8 wells) as a function of cell number in wells. Methylene blue (*a*) and Giemsa (*b*) staining.

Before staining with Giemsa stain, cell suspensions of different density in DMEM with 10% fetal calf serum was added into 96-well plates (200 µl). The first vertical row was the control (no cell suspension). The plates were incubated at 37°C and 5% CO_2 for 24 h and fixed by adding 50 µl cold fresh 2.5% glutaraldehyde. After 30-min fixation at 4°C the fixative was removed, the wells were washed twice with cold Hanks' solution, and filled with 100 µl Giemsa stain specifically binding chromatin diluted 1:50 directly

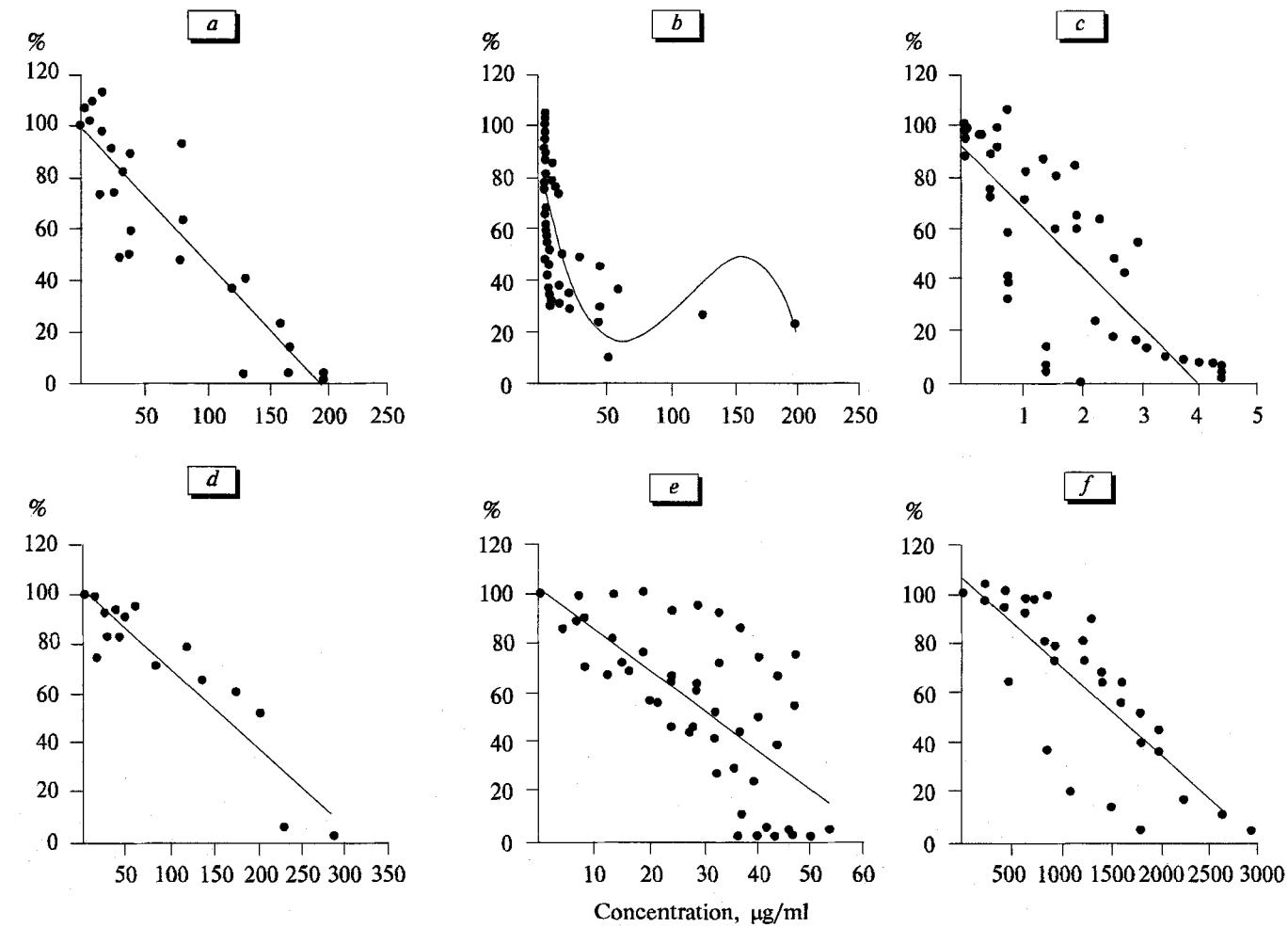

Fig. 2. Cell survival as a function of drug dose. Methylene blue staining. Ordinate: optical density, % of control (without treatment). *a*) acrylamide (*n*=3), *b*) cycloheximide (*n*=4), *c*) sanguiritrine (*n*=10), *d*) sodium dodecyl sulfate (*n*=2), *e*) streptomycin (*n*=5), and *e*) ethanol (*n*=4).

before use. The cells were incubated with the stain for 3 h at 37°C, after which the stain was removed, wells were washed twice with Hanks' solution and incubated with 100 μl eluting solution (0.1 M NaH_2PO_4: C_2H_5OH, 1:1) for 15 min at 18-20°C and at constant stirring. Optical density of eluting solution was measured on an EFOS 9305 photometer at 620 nm.

RESULTS

Standard cultures of postnatal diploid human fibroblasts IMG 1540 and IMG 1398 used in our study were characterized by oriented growth with the formation of confluent monolayer for at least 30 passages. Under standard conditions the density of cultured cells reached 10^5 cells/cm^2 after 14 days. Microscopy showed typical fibroblast-like spindle cells, not apparently differing from other normal human fibroblasts stored at Cell Bank of Medical Genetics Research Center. All experiments were performed on these cells within 10-30 passages.

The intensity of methylene blue and Giemsa staining was directly proportionate to the number of cells in wells (Fig. 1, *a, b*). After detecting this relationship, we evaluated the biological effects of BAS. When different doses of acrylamide, streptomycin, cycloheximide, sodium dodecyl sulfate, sanguiritrine, and ethanol were added, typical dose-effect curves were plotted for all drugs except cycloheximide (Fig. 2), which allowed us to estimate LD_{50} for these agents (*e. g.* about 2 μg/ml for sanguiritrine, mean of 10 experiments). Similar results were obtained in experiments with Giemsa staining.

Biological effects of streptomycin, acrylamide, sodium dodecyl sulfate, and ethanol were qualitatively similar, while studies of cycloheximide revealed a principally different biphasic relationship (Fig. 2, *b*): about 20% cells in the culture remained viable even at high concentrations of the antibiotic, which may be explained by so-called polyribosome "freezing".

Microscopy showed that at sanguiritrine concentration above 4 μg/ml the number of dead cells, on which green sediment of the drug precipitated, drastically increased. Therefore, morphological analysis of cell culture after exposure to BAS is an obligatory element of this test system.

Hence, the proposed test system can be used for evaluating the cytotoxic effects of BAS acting via different mechanisms, from direct damage to DNA and polyribosome complex blockade to membranotropic and denaturing effects.

REFERENCES

1. *All-Union Collection of Cell Cultures. Catalogue,* Leningrad (1991), pp. 48-51.
2. K. N. Grinberg, V. I. Kukharenko, V. N. Lyashko, *et al., Methods of Cell Culturing* [in Russian], Leningrad (1988), pp. 250-257.
3. B. Romeis, *Microscopic Technique* [in Russian], Moscow (1953), pp. 471-473.
4. *Testing of Drugs for External Use in Human Skin Cell Culture* [in Russian], Moscow (1996).
5. V. G. Chernikov, T. B. Krokhina, S. S. Shishkin, *et al., Biomedical Technologies.* Proceedings of Vsesoyuzn. Instituta Lekarstvennykh Rastenii (Rossiisk. Akad. Sel'skokhozyaistvennykh Nauk), Moscow (1998), Vol. 10, pp. 53-57.
6. L. Chuniaud, M. Dessante, F. Chantoux, *et al., Clin. Chim. Acta,* **256,** No. 2, 103-114 (1996).
7. S. Dhar, P. Nygren, K. Csoka, *et al., Brit. J. Cancer,* **74,** No. 6, 888-896 (1996).
8. P. Knox, P. F. Uphill, J. R. Fry, *et al., Food Chem. Toxicol.,* **24,** No. 6/7, 457-463 (1986).
9. R. J. Riddell, R. H. Clothier, and M. Balls, *Ibid.,* 469-471.
10. P. Rivalland, K. Vie, L. Coiffard, and Y. De Roeck Holtzhauer, *Pharm. Acta Helv.,* **69,** No. 3, 159-162 (1994).
11. D. Scudiero, R. Shoemaker, K. Paull, *et al., Cancer Res.,* **48,** 4827-4833 (1988).
12. P. Skehan, R. Storeng, D. Ascudiero, *et al., J. Natl. Cancer Inst.,* **82,** 1107-1112 (1990).