WORLD HEALTH ORGANIZATION

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

TRANSFORMATION ASSAY OF ESTABLISHED CELL LINES: MECHANISMS AND APPLICATION

Proceedings of a workshop organized by IARC in collaboration with the US National Cancer Institute and the US Environmental Protection Agency, held in Lyon, 15-17 February 1984

EDITORS

T. KAKUNAGA & H. YAMASAKI

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The International Agency for Research on Cancer (IARC) was established in 1965 by the World Health Assembly, as an independently financed organization within the framework of the World Health Organization. The headquarters of the Agency are at Lyon, France.

The Agency conducts a programme of research concentrating particularly on the epidemiology of cancer and the study of potential carcinogens in the human environment. Its field studies are supplemented by biological and chemical research carried out in the Agency's laboratories in Lyon and, through collaborative research agreements, in national research institutions in many countries. The Agency also conducts a programme for the education and training of personnel for cancer research.

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FOREWORD

No single short-term test can predict all classes of carcinogen. Many short-term tests are designed to measure the ability of chemicals to cause genetic damage, i.e., mutation and chromosomal effects. Cell transformation occupies a unique position among carcinogen tests, since this assay may be capable of detecting nongenetic activity as well as genetic activity of chemicals. Since both genetic and nongenetic mechanisms are probably involved in carcinogenesis, cell transformation assays may detect a wide range of carcinogens. In addition, the system represents a good model for studying molecular and cellular mechanisms of carcinogenesis.

It is the hope of the Agency that the discussions and recommendations prepared during the workshop of which this publication is the outcome represent the 'state of the art' of current understanding of mechanisms of cell transformation and will promote international coordination in use of the assay as a carcinogen-screening test.

The Workshop was organized in collaboration with the US National Cancer Institute (NCI) and the US Environmental Protection Agency (EPA). The Agency expresses its sincere thanks to all those who helped in organizing the Workshop, especially Dr T. Kakunaga (NCI) and Dr S. Nesnow (EPA).

L. Tomatis, M.D.
Director,
IARC

INTRODUCTION

Although it has long been recognized that the endpoint of in-vitro cell transformation tests has direct relevance to carcinogenesis, their use for screening potential carcinogens has not been fully exploited. This limited use of in-vitro cell transformation is due mainly to the following factors: (1) our knowledge of molecular and cellular mechanisms is still limited; (2) cell transformation assays are technically more difficult than most other in-vitro screening tests; (3) scoring of morphologically transformed foci has been somewhat subjective; (4) the method has not been validated internationally as a screening test for potential carcinogens.

A Working Group was therefore convened to discuss molecular and cellular mechanisms of cell transformation, biological similarities between in-vitro cell transformation and in-vivo carcinogenesis, and the feasibility of screening environmental carcinogens using in-vitro transformation assays in established cell lines. The Working Group identified the methodological and technical problems associated with these assays, prepared recommendations for practical procedures in the performance of cell transformation assays, and developed standard methods for scoring morphologically transformed foci.

The Working Group discussed the problems involved in the use of only two established cell lines, BALB/c 3T3 (3T3) and C3H/10T½ (10T½), since these two systems share several common features. In preparing the recommendations for criteria to be used in scoring transformed foci, the Working Group actually examined fixed and stained petri dishes containing various foci. Since, in the future, the use of cell transformation systems for screening of potential carcinogens will doubtless increase, and the assay methods improve accordingly, the recommendations of the present Working Group may be updated periodically as the state of the art advances.

The Editors

PARTICIPANTS

G.A. Bannikov[1]	Carcinogenesis Mechanisms Laboratory, All-Union Cancer Research Center, 6 Kashirskoye shosse, 115487 Moscow, USSR
J.M. Bechet	International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 8, France
J. Bertram	Department of Experimental Therapeutics, Roswell Park Memorial Institute, Elm Street, Buffalo, NY 14263, USA
I. Chouroulinkov	Institut de Recherches Scientifiques sur le Cancer, BP 8, 94802 Villejuif Cedex, France
E. Cortesi	Istituto Patologia Generale, III Cattedra, Universita di Roma, viale Regina Elena 324, 00161 Roma, Italy
T. Enomoto	International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 8, France
L. Haroun	International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 8, France
J.C.M. van der Hoeven	Notox, Pathobiology Research BV, Hambakenwetering 31, 5231 DD's Hertogenbosh, The Netherlands
T. Kakunaga	Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita-shi, Osaka, Japan
T. Kuroki	Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai Minato-ku, Tokyo 108, Japan
J.R. Landolph	University of Southern California School of Medicine, Cancer Research Laboratory, 1303 North Mission Road, Los Angeles, CA 90033, USA

[1] Unable to attend

J.B. Little	Laboratory of Radiobiology, Harvard University, School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA
R. Montesano	International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
S. Nesnow[1]	Chief, Carcinogenesis and Metabolism Branch (MD-68), Health Effects Research Laboratory, Research Triangle Park, NC 27711, USA
R.F. Newbold	Chemical Carcinogenesis Division, Institute of Cancer Research, Pollards Wood Research Station, Chalfont St Giles, Buckinghamshire, HP8 4SP, UK
L. Schechtman	Microbiological Associates, 5221 River Road, Bethesda, MD 20015, USA
A. Sivak	Bio/Medical Sciences Section, Arthur D. Little, Inc., Cambridge, MA 02140, USA
M. Umeda	Tissue Culture Laboratory, Yokohama City University, School of Medicine, 2-33 Urafune-cho, Minami-ku, Yokohama, Japan
H. Yamasaki	International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 08, France

IARC Technical Assistants

A.M. Aguelon
C. Fuchez

CELLULAR AND MOLECULAR MECHANISMS OF CELL TRANSFORMATION

CELLULAR MECHANISMS OF ONCOGENIC TRANSFORMATION *IN VITRO*

J.B. Little

Department of Cancer Biology,
Harvard University School of Public Health,
Boston, MA, USA

INTRODUCTION

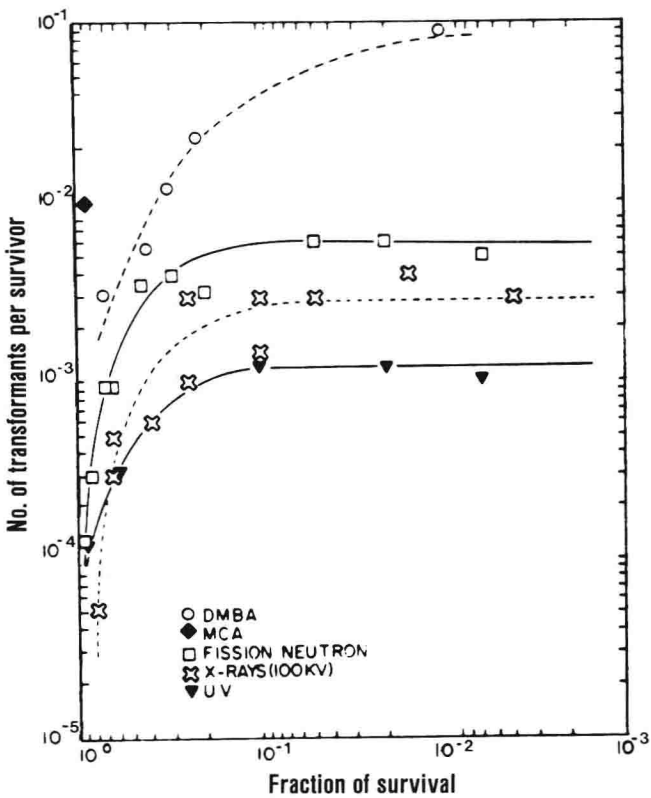
During the past ten years, established mouse embryo fibroblast cell lines that are highly sensitive to the post-confluence inhibition of cell division have been used widely to study malignant transformation *in vitro* by physical and chemical agents. These cell lines offer several advantages over other transformation systems for such studies. Since they are established lines rather than primary cultures, the cells can be cloned and grown in large quantities, and cells from the same cloned population can be used by many laboratories. Thus, the reproducibility of results obtained with these cell lines among different laboratories has in general been very high. Transformed cells are readily identifiable and can be scored quantitatively by morphological criteria in a focus assay. Although spontaneous transformation may occur in nontreated cells, it can generally be stabilized by the systematic maintenance of culture conditions, including the passaging technique and the careful selection of serum to supplement the growth medium.

This chapter reviews some of the cellular phenomena associated with the transformation of established mouse embryo fibroblast-derived cell lines, and particularly those factors that may influence their use in assays of the carcinogenic potential of environmental chemicals. The review focuses upon the two cell systems in current use: the C3H mouse 10T½ (10T½) cell line originally isolated by Reznikoff *et al.* (1973a) and the cloned cell lines derived by Kakunaga (1973) from the A31 subclone of mouse BALB/c 3T3 cells (3T3). It is important to remember that these are immortalized lines of aneuploid cells, which have therefore already undergone some of the changes associated with transformation. Thus, we are actually measuring the conversion of an immortal cell which is phenotypically normal to one which is phenotypically transformed in terms of its morphological and growth characteristics, its ability to grow under anchorage-independent conditions and its ability to form nonregressing tumours upon injection into syngeneic hosts. Although the process of immortalization occurs readily in rodent cells, it appears to be a rare phenomenon in human diploid cells, and the latter is very difficult to achieve experimentally. Some observations on the transformation of human diploid fibroblasts are presented at the end of this chapter, and their relevance to rodent cell transformation assays is discussed.

DOSE-RESPONSE RELATIONSHIPS

The dose-response curves for the induction of transformation by a wide variety of physical and chemical carcinogens shows a similar shape. They are characterized by a rapid increase in the yield of transformants with increasing dose in the lower dose regions, followed by a plateau at higher doses where the yield of transformants reaches a limiting value. These characteristics are shown graphically in Figure 1, in which dose-response curves are presented for exposure to 7,12-dimethylbenz[*a*]anthracene (DMBA), ultraviolet (UV) light, X-irradiation and fission spectrum neutrons. Although the shapes of the dose-response curves are similar, they differ significantly in terms of the transformation frequency at which the plateau is reached. For example, the plateau for neutron exposure is two to three times higher than that for X-ray exposure, whereas the limiting transformation frequency for DMBA appears to be nearly two orders of magnitude higher than that for UV light or X-rays. A possible mechanism for the limiting yield of transformants at higher doses of carcinogen is discussed below.

Figure 1. Dose-response curves for transformation of C3H mouse 10T½ cells by various physical and chemical carcinogens. Reproduced, with permission, from Yang and Tobias (1980), in which the sources of the data are given.



Polycyclic aromatic hydrocarbons have, in general, proven to be considerably more potent than either UV light or ionizing radiation in inducing transformation. As can be seen in Figure 1, the frequency of transformation induced by a noncytotoxic dose of 3-methylcholanthrene (MCA) was considerably higher than the maximum transformation frequency induced by highly cytotoxic doses of X-rays. Certain alkylating agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, have proven to be very weak inducers of transformation in 10T½ cells. The significance of such variations in potency *in vitro* in terms of the carcinogenic potential of these agents in human populations remains to be elucidated. Two other preliminary observations are, however, of interest in this regard. We have noted (Terzaghi & Little, unpublished observations) that a higher frequency of aggressive-looking type III transformed foci was induced in cultures treated with polycyclic aromatic hydrocarbons [benzo[*a*]pyrene (BaP) and MCA] than in cultures treated with X-rays. When Hahn (1980) examined the tumorigenic potential in syngeneic mice of cells isolated from type III foci induced by either MCA or X-rays, he found that many fewer cells from MCA-transformed type III foci were required to produce a 50% tumour take than from type III foci transformed by X-rays. Although this result may have occurred by chance (a relatively small number of cell lines derived from specific foci were examined in this study), the suggestion that cells transformed phenotypically by different carcinogens may differ in their malignant potential deserves further investigation.

A final characteristic of note is the unusually high transformation frequencies that may be observed *in vitro* when the results are expressed in terms of transformation frequency per viable treated cell, as in Figure 1. As can be seen in this figure, the transformation frequency for DMBA reached 10%; transformation frequencies of 1-5% have frequently been recorded in rodent cells treated with various chemical carcinogens. Such frequencies are very high in comparison with those observed for the induction by potent mutagens of single gene mutations in mammalian cells, as well as with the apparently rare occurrence of cancer within treated cell populations *in vivo*.

DEPENDENCE OF TRANSFORMATION FREQUENCY ON INITIAL TREATED CELL DENSITY

In the initial studies of transformation of 10T½ cells by chemical carcinogens and radiation, the dependence of the calculated transformation frequency per viable cell upon the initial number of treated, viable cells per dish was examined (Reznikoff *et al.*, 1973b; Terzaghi & Little, 1974). The results for X-ray transformation (Terzaghi & Little, 1974) indicated that, whereas the transformation frequency appeared to be relatively constant, with viable cell densities of 100 to 400 cells per 100-mm dish, this frequency declined markedly with higher initial seeding densities.

The results for transformation by MCA (Reznikoff *et al.*, 1973b) differed significantly: the lowest cell density studied was 100 viable cells per 100-mm dish (500 total cells/dish); in this case, the transformation frequency declined markedly with all higher initial seeding densities.

This phenomenon was initially interpreted as reflecting the requirement for a minimum number of rounds of cell division for the expression of transformation (Terzaghi & Little, 1976); at higher initial seeding densities, fewer rounds of cell division would ensue prior to the cultures becoming confluent. Shortly thereafter, however, other findings brought this interpretation into question. Preliminary studies of X-ray transformation in 10T½ cells seeded at very low cell densities indicated an unexpectedly steep rise in transformation frequency with initial cell numbers below 100 cells per dish (Little, 1977a). A similar phenomenon was observed in X-irradiated 3T3 cells (Little, 1977a, 1979); in these experiments, the calculated transformation frequency was highly dependent on the initial number of viable cells seeded through a wide range of cell concentrations.

These results were of particular interest in light of a previous and largely ignored finding reported by Mondal and Heidelberger (1970). These workers found that when single cells of a mouse ventral prostate-derived fibroblastic cell line were treated with MCA and the progeny replated during exponential growth, one or two transformed foci developed in all viable dishes. Sometimes mistakenly referred to as indicating '100% transformation', these results actually demonstrate that, under certain conditions, a single treated cell has a high probability of yielding one or more transformed, tumorigenic descendants.

These findings led Kennedy *et al.* (1980a) and Fernandez *et al.* (1980), independently, to carry out a systematic evaluation of the influence of initial seeded cell density on the yield of transformants by X-rays and MCA, respectively. Kennedy *et al.* (1980a) found that the ultimate yield of transformed foci per dish was largely independent of the initial cell density over the range of 1 to 300 viable cells seeded. In other words, the calculated transformation frequency per viable cell was directly related to the initial number of viable cells present in the dish (Fig. 2). Furthermore, when treated cells that were allowed to proliferate to confluence were suspended, reseeded at widely varying cell densities and then allowed to express transformation, an approximately constant yield of foci per dish was again observed, despite a four-fold variation in the number of cells reseeded. These results are shown in Figure 3. The results of these experiments indicate that transformed foci arise in 20-40% of dishes seeded with a single viable cell treated with 600 rads of X-rays alone, whereas up to 80% of dishes seeded with single cells treated with X-rays and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) contain transformed foci (Kennedy & Little, 1980). It is important to remember that the single viable cell proliferates until a normal confluent monolayer is reached; one or more transformed foci develop over this monolayer several weeks later.